PC[redacted]

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For [redacted]ing Office use only

International Application No. 10/009478

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 338-110PCT

Box No. I TITLE OF INVENTION

Modulating Cell Survival by Modulating Huntingtin Function

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

University of British Columbia
Industry Liaison Office
111-2386 East Mall
Vancouver, British Columbia V6T 1Z3
Canada

Telephone No.

Facsimile No.

Teleprinter No.

Applicant's registration No. with the Office

State *(that is, country)* of nationality: CA

State *(that is, country)* of residence: CA

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Michael R. Hayden
4484 West 7th Avenue
Vancouver, British Columbia V6R 1W9
Canada

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence: CA

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MBM & Co.
P.O. Box 809, Station B
Ottawa, Ontario KIP 5P9
Canada

Telephone No. 613-567-0762

Facsimile No. 613-563-7671

Teleprinter No.

Agent's registration No. with the Office

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Abigail S. Hackam
105 West 39th St., Apt. 1219
Baltimore, Maryland 21210
United States of America

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Blair R. Leavitt
4008 West 32nd Ave.
Vancouver, British Columbia V6S 1Z6
Canada

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence: CA

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION [redacted]TATES *Mark the applicable check-boxes belo[redacted]east one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

☒ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ EP **European Patent:** AT Austria, BE Belgium, CH & LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, TR Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒ AE United Arab Emirates
☒ AG Antigua and Barbuda
☒ AL Albania
☒ AM Armenia
☒ AT Austria
☒ AU Australia
☒ AZ Azerbaijan
☒ BA Bosnia and Herzegovina
☒ BB Barbados
☒ BG Bulgaria
☒ BR Brazil
☒ BY Belarus
☒ BZ Belize
☒ CA Canada
☒ CH & LI Switzerland and Liechtenstein
☒ CN China
☒ CO Colombia
☒ CR Costa Rica
☒ CU Cuba
☒ CZ Czech Republic
☒ DE Germany
☒ DK Denmark
☒ DM Dominica
☒ DZ Algeria
☒ EE Estonia
☒ ES Spain
☒ FI Finland
☒ GB United Kingdom
☒ GD Grenada
☒ GE Georgia
☒ GH Ghana
☒ GM Gambia
☒ HR Croatia
☒ HU Hungary
☒ ID Indonesia
☒ IL Israel
☒ IN India
☒ IS Iceland
☒ JP Japan
☒ KE Kenya
☒ KG Kyrgyzstan
☒ KP Democratic People's Republic of Korea
☒ KR Republic of Korea
☒ KZ Kazakhstan
☒ LC Saint Lucia
☒ LK Sri Lanka
☒ LR Liberia
☒ LS Lesotho
☒ LT Lithuania
☒ LU Luxembourg
☒ LV Latvia
☒ MA Morocco
☒ MD Republic of Moldova
☒ MG Madagascar
☒ MK The former Yugoslav Republic of Macedonia
☒ MN Mongolia
☒ MW Malawi
☒ MX Mexico
☒ MZ Mozambique
☒ NO Norway
☒ NZ New Zealand
☒ PL Poland
☒ PT Portugal
☒ RO Romania
☒ RU Russian Federation
☒ SD Sudan
☒ SE Sweden
☒ SG Singapore
☒ SI Slovenia
☒ SK Slovakia
☒ SL Sierra Leone
☒ TJ Tajikistan
☒ TM Turkmenistan
☒ TR Turkey
☒ TT Trinidad and Tobago
☒ TZ United Republic of Tanzania
☒ UA Ukraine
☒ UG Uganda
☒ US United States of America
☒ UZ Uzbekistan
☒ VN Viet Nam
☒ YU Yugoslavia
☒ ZA South Africa
☒ ZW Zimbabwe

Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet:

☒ all member states ☐ ☐
☐ ☐ ☐

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CL

The priority of the following earlier application(s) is hereby claimed:

	Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1)	13/04/2000	2,305,088	CA		
item (2)	12/12/2000	2,326,543	CA		
item (3)					
item (4)					
item (5)					

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☒ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box

* *Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii))*: . . . .

. . . . . .

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / . . . . . .

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following declarations are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*: Number of declarations

☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application contains:

(a) the following number of sheets in paper form:

request (including declaration sheets)	: 5
description (excluding sequence listing part)	: 48
claims	: 4
abstract	: 1
drawings	: 18
Sub-total number of sheets	: 76
sequence listing part of description *(actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (b) below)*	:
Total number of sheets	:

(b) sequence listing part of description filed in **computer readable form**

(i) ☐ only (under Section 801(a)(i))

(ii) ☐ in addition to being filed in paper form (under Section 801(a)(ii))

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained *(additional copies to be indicated under item 9(ii), in right column)*: .....

This international application is accompanied by the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*:

	Number of items
1. ☐ fee calculation sheet	:
2. ☐ original separate power of attorney	:
3. ☐ original general power of attorney	:
4. ☐ copy of general power of attorney; reference number, if any:	:
5. ☐ statement explaining lack of signature	:
6. ☐ priority document(s) identified in Box No. VI as item(s):	:
7. ☐ translation of international application into *(language)*:	:
8. ☐ separate indications concerning deposited microorganism or other biological material	:
9. ☐ sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other))	
(i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application)	:
(ii) ☐ *(only where check-box (b)(i) or (b)(ii) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter*	:
(iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column	:
10. ☐ other *(specify)*:	:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

MBM + Co.

MBM & Co. (Margaret Swain, Partner)

For receiving Office use only

1. Date of actual receipt of the purported international application:

2. Drawings:
☐ received:
☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA /

6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
MBM & CO.
P.O. Box 809
Station B
Ottawa, Ontario K1P 5P9
CANADA

Applicant's or agent's file reference 338-110PCT	Date of mailing *(day/month/year)* 27/08/2001 **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/CA 01/ 00495	International filing date *(day/month/year)* 12/04/2001
Applicant UNIVERSITY OF BRITISH COLUMBIA et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Katarina Faux

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
338-110PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 01/ 00495	12/04/2001	13/04/2000
Applicant		
UNIVERSITY OF BRITISH COLUMBIA et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 01/00495

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C12N15/87 C12N5/02 A61K38/17 A61K31/7052
A61P25/28 A61P15/08 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K A61P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 60986 A (MERCK FROSST CANADA INC ;UNIV BRITISH COLUMBIA (CA); VALLAINCOURT) 2 December 1999 (1999-12-02) abstract, page 2, Fig. 9, claims ---	1-5, 7-12,15, 16, 20-22, 26-28
X	EP 0 614 977 A (GEN HOSPITAL CORP) 14 September 1994 (1994-09-14) p. 3, summary, pp.9/10, claims ---	1-5,7,8, 12, 15-18, 20-24, 26-30
X	WO 99 45944 A (BURNHAM INST) 16 September 1999 (1999-09-16) pp. 5/6 the whole document --- -/--	3,4,20, 21,28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2001	27/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Stolz, B

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 06545 A (MAX PLANCK GESELLSCHAFT ;LEHRACH HANS (DE); WANKER ERICH (DE); SCH) 11 February 1999 (1999-02-11) pp. 2/3, claims	1
X	KIM M ET AL: "MUTANT HUNTINGTIN EXPRESSION IN CLONAL STRIATAL CELLS: DISSOCIATIONOF INCLUSION FORMATION AND NEURONAL SURVIVAL BY CASPASE INHIBITION" JOURNAL OF NEUROSCIENCE, NEW YORK, NY, US, vol. 19, no. 3, 1 February 1999 (1999-02-01), pages 964-973, XP000973324 ISSN: 0270-6474 the whole document	1
P,X	LEAVITT B.R. ET AL.: "Wild-Type Huntingtin Reduces the Cellular Toxicity of Mutant Huntingtin In Vivo" AM. J. HUM. GENETICS, vol. 68, 2001, pages 313-324, XP001013155 electronically published December 20, 2000 the whole document	1-31
P,X	WO 00 78813 A (UNIV EMORY) 28 December 2000 (2000-12-28) pp. 15/16	1,4,5,7, 8,13,16, 22,24, 28,30
P,X	WO 01 06989 A (MESSER ANNE ;HUSTON JAMES S (US); LECERF JEAN MICHEL (US)) 1 February 2001 (2001-02-01) the whole document	1,4,5,7, 8,13,16, 22,24, 28,30
P,X	WO 01 09613 A (NERI CHRISTIAN ;FOND JEAN DAUSSET CEPH (FR)) 8 February 2001 (2001-02-08) the whole document	1,4,5,7, 22,28
P,X	HEISER VOLKER ET AL: "Inhibition of huntingtin fibrillogenesis by specific antibodies and small molecules: Implications for Huntington's disease therapy" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 12, 6 June 2000 (2000-06-06), pages 6739-6744, XP002155981 ISSN: 0027-8424 the whole document	1,4,13

-/--

International Application No
PCT/CA 01/00495

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	RIGAMONTI D., ET AL.: "Wild-Type Huntingtin Protects from Apoptosis Upstream of Caspase 3" THE JOURNAL OF NEUROSCIENCE, vol. 20, no. 10, 15 May 2000 (2000-05-15), pages 3705-3713, XP002174028 cited in the application the whole document	1-31

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 4, 14, 16, 19, 22, 25, 28, 31 (all partially)

Claims relating to antagonists are only searched as far as anti-huntingtin antibodies, htt antisense molecules or mutant huntingtin are concerned. Claims 4, 14, 16, 19, 22, 25, 28, 31 relate to subject matter defined by having an effect on cell proliferation. The subject matter is structurally undefined and not all of the prior art substances have been tested for this effect. Therefore, a meaningful search for prior art affecting novelty or inventive step is not possible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 01/00495

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1 to 14 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 4, 14, 16, 19, 22, 25, 28, 31 (all partially).
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 01/00495

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9960986 A	02-12-1999	US	6235879 B	22-05-2001
		AU	4212199 A	13-12-1999
		EP	1082336 A	14-03-2001
EP 0614977 A	14-09-1994	AU	676001 B	27-02-1997
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		EP	1061935 A	27-12-2000
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WO 0106989 A	01-02-2001	AU	6234700 A	13-02-2001
WO 0109613 A	08-02-2001	FR	2797055 A	02-02-2001

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 01/00495

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C12N15/87 C12N5/02 A61K38/17 A61K31/7052
A61P25/28 A61P15/08 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K A61P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 60986 A (MERCK FROSST CANADA INC ;UNIV BRITISH COLUMBIA (CA); VALLAINCOURT) 2 December 1999 (1999-12-02) abstract, page 2, Fig. 9, claims	1-5, 7-12,15, 16, 20-22, 26-28
X	EP 0 614 977 A (GEN HOSPITAL CORP) 14 September 1994 (1994-09-14) p. 3, summary, pp.9/10, claims	1-5,7,8, 12, 15-18, 20-24, 26-30
X	WO 99 45944 A (BURNHAM INST) 16 September 1999 (1999-09-16) pp. 5/6 the whole document	3,4,20, 21,28

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2001	27/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Stolz, B

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/CA 01/00495

C.(Continuation) DOCUMENTS CONSIDERE[redacted]E RELEVANT

C ory °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 06545 A (MAX PLANCK GESELLSCHAFT ;LEHRACH HANS (DE); WANKER ERICH (DE); SCH) 11 February 1999 (1999-02-11) pp. 2/3, claims	1
X	KIM M ET AL: "MUTANT HUNTINGTIN EXPRESSION IN CLONAL STRIATAL CELLS: DISSOCIATIONOF INCLUSION FORMATION AND NEURONAL SURVIVAL BY CASPASE INHIBITION" JOURNAL OF NEUROSCIENCE, NEW YORK, NY, US, vol. 19, no. 3, 1 February 1999 (1999-02-01), pages 964-973, XP000973324 ISSN: 0270-6474 the whole document	1
P,X	LEAVITT B.R. ET AL.: "Wild-Type Huntingtin Reduces the Cellular Toxicity of Mutant Huntingtin In Vivo" AM. J. HUM. GENETICS, vol. 68, 2001, pages 313-324, XP001013155 electronically published December 20, 2000 the whole document	1-31
P,X	WO 00 78813 A (UNIV EMORY) 28 December 2000 (2000-12-28) pp. 15/16	1,4,5,7, 8,13,16, 22,24, 28,30
P,X	WO 01 06989 A (MESSER ANNE ;HUSTON JAMES S (US); LECERF JEAN MICHEL (US)) 1 February 2001 (2001-02-01) the whole document	1,4,5,7, 8,13,16, 22,24, 28,30
P,X	WO 01 09613 A (NERI CHRISTIAN ;FOND JEAN DAUSSET CEPH (FR)) 8 February 2001 (2001-02-08) the whole document	1,4,5,7, 22,28
P,X	HEISER VOLKER ET AL: "Inhibition of huntingtin fibrillogenesis by specific antibodies and small molecules: Implications for Huntington's disease therapy" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 12, 6 June 2000 (2000-06-06), pages 6739-6744, XP002155981 ISSN: 0027-8424 the whole document	1,4,13

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INTERNATIONAL SEARCH REPORT

Int[illegible]nal Application No
PCT/CA 01/00495

C.(Continuation) DOCUMENTS CONSIDE[illegible]O BE RELEVANT

[illegible]tegory °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	RIGAMONTI D., ET AL.: "Wild-Type Huntingtin Protects from Apoptosis Upstream of Caspase 3" THE JOURNAL OF NEUROSCIENCE, vol. 20, no. 10, 15 May 2000 (2000-05-15), pages 3705-3713, XP002174028 cited in the application the whole document	1-31

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 4, 14, 16, 19, 22, 25, 28, 31 (all partially)

Claims relating to antagonists are only searched as far as anti-huntingtin antibodies, htt antisense molecules or mutant huntingtin are concerned. Claims 4, 14, 16, 19, 22, 25, 28, 31 relate to subject matter defined by having an effect on cell proliferation. The subject matter is structurally undefined and not all of the prior art substances have been tested for this effect. Therefore, a meaningful search for prior art affecting novelty or inventive step is not possible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 01/00495

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9960986 A	02-12-1999	US 6235879 B	22-05-2001
		AU 4212199 A	13-12-1999
		EP 1082336 A	14-03-2001
EP 0614977 A	14-09-1994	AU 676001 B	27-02-1997
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WO 0109613 A	08-02-2001	FR 2797055 A	02-02-2001

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/79283 A1

(54) Title: MODULATING CELL SURVIVAL BY MODULATING HUNTINGTIN FUNCTION

(57) Abstract: The present invention provides a means of modulating cell survival by modulating wild-type huntingtin protein function by administration of wild-type huntingtin protein, biologically active fragments thereof and/or antagonists of wild-type huntingtin. Accordingly, the present invention also provides biologically active fragments of wild-type huntingtin, antagonists of wild-type huntingtin and nucleic acid sequences encoding the biologically active fragments and peptide antagonists, and their use to modulate cell survival. In particular, the invention provides for means to activate or attenuate cell death within tissue, in order to facilitate the treatment of conditions where there is a dysregulation of cell death or cellular proliferation. Therapeutic application of this invention pertains to diseases and disorders including, but not limited to, Huntington disease, neurodegenerative diseases, stroke, and cancer.

MODULATING CELL SURVIVAL BY MODULATING HUNTINGTIN FUNCTION

FIELD OF INVENTION

The invention pertains to the field of medicine, specifically to therapeutic or preventative compositions for use in the treatment of mammalian conditions characterised by a dysregulation of cell death or cellular proliferation.

BACKGROUND OF THE INVENTION

Huntington disease is a neurodegenerative disease caused by mutation of the huntingtin protein (known as huntingtin or htt). The physiological role of the huntingtin protein is currently unknown. HD (Huntington's Disease) is a devastating neurological disease which usually presents in mid adult life, affects approximately 1 in 10,000 individuals (Hayden 1981), and results in psychiatric disturbance, involuntary movement disorder, and cognitive decline associated with inexorable progression to death, typically 17 years following onset.

A recent epidemiological study shows that there is a lower incidence of cancer among patients with HD which appears to be related to intrinsic biologic factors (Sorensen *et al.* (1999) *Cancer* **86**, 1342-1346). A possible explanation for this may be that the mutant huntingtin protects against cancer by inducing or increasing the rate of naturally occurring apoptosis in preneoplastic cells. This further implicates that the induced or increased apoptosis, resulting from the presence of mutant huntingtin, is a factor in the generation of HD. This theory is further supported by a cell culture study which demonstrated that the expression of truncated N-terminal huntingtin containing the expanded polyglutamine caused a repeat length- and dose-dependent increase in the formation of aggregates and cell death (Martindale et al. (1998) *Nat. Genet.* 18,150-154; Wang *et al.* (1999) *Neuroreport.* **10**, 2435-2438). The researchers observed that caspases were activated, and the death substrates of caspases, lamin B and inhibitor of caspase-activated DNase (ICAD), were cleaved in this cell death process. These findings suggest that huntingtin

with the polyglutamine expansion was responsible for cell death induction and that this cell death is mediated by caspases.

Apoptosis is also called "programmed cell death" or "cell suicide". (Krammer *et al.* (1991) "Apoptosis in the APO-1 System", Apoptosis: The Molecular Basis of Cell Death, pp. 87-99 Cold Spring Harbor Laboratory Press). When the normal function of apoptosis goes awry, the cause or the result can be one of a number of diseases, including: cancer, viral infections, autoimmune disease/allergies, neurodegeneration or cardiovascular diseases. In HD, it is not known how the mutant gene that is widely expressed results in selective neuronal death. Further, sequence analysis has revealed no obvious homology to other known genes and no structural motifs or functional domains have been identified which clearly provide insights into its function. In particular, the question of how these widely expressed genes cause selective neuronal death remains unanswered. In addition, the role of wild-type huntingtin protein in the normal life cycle of cells, involving the apoptotic pathway and cellular proliferation, throughout the body of an organism, remains to be determined.

Mice heterozygous for targeted disruption of the HD gene express half the normal levels of wild-type htt, and have previously been shown to develop neuronal degeneration in the basal ganglia (Nasir *et al.* (1995) *Cell* **81**, 811-823; O'Kusky *et al.* (1999) *Brain Res.* **818**, 468-479. Wild-type htt has also recently been shown to protect cells from apoptotic stimuli *in vitro* (Rigamonti *et al.* (2000) *J. Neurosci.* **20**, 3705-3713).

The present invention establishes a biological function for huntingtin protein and/or biologically active fragments thereof, in addition to nucleic acid sequences encoding such proteins.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a means of modulating cell survival by modulating the function of huntingtin protein, and/or biologically active fragments thereof. In particular, the invention provides a means to modulate the cellular balance between proliferation and death in order to facilitate the treatment of conditions where there is a dysregulation of apoptosis or cellular proliferation. Therapeutic application of

this invention pertains to diseases and disorders characterised by an increase in apoptosis including, but not limited to, Huntington disease, neurodegenerative diseases and stroke, in addition to diseases characterised by dysregulated cellular proliferation, such as cancer.

According to one aspect of the present invention there is provided nucleic acids encoding huntingtin protein, or fragment thereof, and their use to modulate cell survival.

According to another aspect of the present invention there is provided a use of huntingtin protein, or biologically active fragments thereof that will increase the level of huntingtin protein function within cellular material, for the treatment of conditions characterised by dysregulated cell death.

According to another aspect of the present invention there is provided a use of antagonists to huntingtin protein to decrease huntingtin protein function in the treatment of conditions characterised by dysregulation of cellular proliferation

According to one aspect of the present invention there is provided pharmaceutical compositions comprising huntingtin, a biologically active fragment of huntingtin, or a combination thereof and a pharmaceutically acceptable diluent or excipient.

According to another aspect of the present invention there is provided a use of huntingtin, a biologically active fragment of huntingtin, or a combination thereof for the preparation of a medicament for the treatment of conditions characterised by dysregulation of cell death or cell proliferation.

According to another aspect of the present invention there is provided compositions comprising a nucleic acid encoding huntingtin protein, a nucleic acid encoding an active fragment of huntingtin, or a combination thereof, and a pharmaceutically acceptable diluent or excipient, wherein said compositions are used for the treatment of conditions characterised by dysregulation of cell death and wherein said compositions attenuate cell death and/or increase cell proliferation.

According to another aspect of the present invention there is provided compositions comprising antagonists to huntingtin protein and a pharmaceutically acceptable diluent or excipient, wherein said compositions are used for the treatment of conditions characterised by dysregulation of cellular proliferation and wherein said compositions decrease cellular proliferation and/or activate apoptosis.

According to another aspect of the present invention there is provided an assay for screening for molecules having an anti-proliferative activity comprising the steps of transfecting NIH3T3 cells with huntingin, adding a candidate molecule to the transfected cells and comparing proliferation of the transfected cells treated with the candidate molecule with proliferation of the transfected cells in the absence of the candidate molecule, wherein an anti-proliferative effect is found where there is a decrease in proliferation of the treated and transfected cells in comparison to the untreated transfected cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a schematic representation of the balance between normal cell proliferation and cell death. An imbalance will result in abnormal proliferation or abnormal cell death as is observed in various diseases and disorders.

Figure 2 depicts the testicular morphology of yeast artificial chromosome (YAC) rescued huntingtin knockout mice. The YACs used to rescue mice contain the huntingtin gene with 18, 46 or 72 CAG repeats. The normal number of CAG repeats found in humans without HD is 18. Greater numbers of CAG repeats are associated with HD development, with 46 being an intermediate number and 72 being a high number of repeats. Semi-thin sections of testes stained with toluidine blue from 8 month old mice reveal the gross testicular morphology of mice with varying amounts of endogenous huntingtin rescued with YAC18 (a, b, c), YAC46 (d, e, f), and YAC72 (g, h, i) transgenes. Massive degeneration of spermatocytes is shown, and this novel cell death phenotype is CAG repeat size-dependent and is modulated by the level of endogenous huntingtin. The cell death is most pronounced in YAC72 rescued knockout mice, intermediate in YAC46 knockout mice and not present in YAC18 rescued mice.

Figure 3 depicts protein aggregates in YAC72 rescued huntingtin knockout mice. Ultrastructural analysis of the testes of YAC72 mice lacking endogenous huntingtin revealed the presence of abnormal aggregates of intracellular protein (arrows) within spermatids, sertoli cells and sperm tails. The composition of these protein aggregates is currently unknown, but they resemble the ultrastructural appearance of huntingtin aggregates found in human HD brain tissue. Ectopic microtubule bundles and manchettes were also identified (arrows).

Figure 4 depicts electron microscopic (EM) analysis of degenerating testicular cells from YAC72 rescued huntingtin knockout mice. Ultrastructural analysis of testes from YAC72 mice nullizygous for endogenous huntingtin reveals massive cell death of spermatids, phagocytosis of degenerating cells and formation of multinucleated giant cells.

Figure 5 demonstrates that the C-terminus of huntingtin protein is anti-apoptotic. Using the methylthiazol tetrazolium (MTT) assay to measure cell viability, it is shown that expression of the C-terminus (C-ter) of huntingtin protects HEK 293T cells from tamoxifen induced cell death when compared with expression of β-galactosidase (LacZ) as a control.

Figure 6 demonstrates that the C-terminus of huntingtin protein confers protection against huntingtin toxicity in NT2 cells. Mutant huntingtin protein (HD138) was co-transfected with control protein, pyruvate kinase (PK), or with huntingtin C-terminus (C-ter) and cell death in response to tamoxifen stimulus was measured. Expression of the C-terminus reduced HD138-dependent cell death ($p = 0.002$).

Figure 7 demonstrates that the C-terminus of huntingtin protein rescues HIP-1 toxicity in NT2 cells. Expression of the C-terminus in NT2 cells reduces HIP-1 mediated cell death, compared with expression of control protein (PK) ($p < 0.01$).

Figure 8 demonstrates that the C-terminus of huntingtin protein reduces mutant huntingtin protein aggregate formation. Expression of the C-terminus (C-ter) reduces the number of aggregates formed by a truncated version of mutant huntingtin protein (1955-128),

compared with coexpression of huntingtin and the LacZ control. Aggregates were induced by tamoxifen stimulus (A) or by HIP-1 expression (B).

Figure 9 shows results of studies with YAC transgenic mice expressing increased levels of wild-type human huntingtin are resistant to neurodegeneration following kainic acid-induced seizures. **a,** Quantification of degenerating hippocampal neurons following kainic acid-induced seizures in mice expressing 2-3 times the endogenous levels of wild-type huntingtin **(212 line)** and littermate controls **(FVB/NJ)**. Average numbers of degenerating neurons per animal identified by Fluoro-Jade labeling are expressed for the CA1, CA3 and total hippocampal regions. Data is expressed as mean +/- SEM with significance determined using a two-tailed students t-test. Images of degenerating neurons (arrows) using Fluoro-Jade labeling **(b.** line 212 20x, **c.** FVB/NJ 20x, **d.** FVB/NJ 100x), silver staining **(e.** line 212 20x, **f.** FVB/NJ 20x, **g.** FVB/NJ 100x), and TUNEL staining **(h.** line 212 20x, **i.** FVB/NJ 20x, **j.** FVB/NJ 100x) within the hippocampus following KA-induced seizures.

Figure 10 shows YAC transgenic mice expressing increased levels of wild-type human huntingtin have decreased caspase-3 activation following kainic acid-induced seizures. Quantification of hippocampal and cerebellar DEVD-ase activity following kainic acid-induced seizures in mice expressing 2-3 times the endogenous levels of wild-type huntingtin **(212 line)** and littermate controls **(FVB/NJ)**. Data is expressed as mean +/- SEM with significance determined using a two-tailed students t-test.

Figure 11 shows rescue of the *Hdh* nullizygous lethal phenotype by YAC transgenes expressing mutant huntingtin. Resultant genotypes for the F2 offspring of a cross between a YAC72 transgene positive, *Hdh* gene heterozygous mouse (+,+/- genotype) and a transgene negative, *Hdh* heterozygous mouse (-,+/- genotype). (a). The upper PCR bands represent the presence or absence of the YAC transgene and the lower bands represent the state of the endogenous *Hdh* gene. The mouse represented in lane two has the YAC72 transgene, but lacks the endogenous *Hdh* gene (+,-/- genotype). This mouse demonstrates that mutant human huntingtin expression form our YAC transgene rescued the *Hdh* nullizygous state. Mice with targeted disruption of the *Hdh* gene were rescued from the embryonic lethal phenotype equally by all three of the YAC transgenes described in this

paper. The F2 offspring of our experimental breedings had the expected 1:2:1 ratio of genotypes for all of the YAC transgenes examined (B). Western blot analysis of huntingtin protein expression (C) confirmed the absence of endogenous htt protein in *Hdh* nullizygous mice (-/-) compared to wild-type (+/+) and demonstrated similar levels of human transgenic huntingtin expression in YAC18, YAC46, and YAC72 rescued *Hdh* nullizygous mice (+,-/-). Average testicular weight and epididymal sperm counts for YAC72+/+, YAC72+/-, and YAC72-/- mice at four months of age are shown in (D) and (E) respectively. YAC72-/- mice had significant testicular atrophy ($p<10^{-5}$) and decreased sperm counts ($p<10^{-5}$) compared to YAC72+/+ and YAC72+/- mice.

Figure 12 shows testicular morphology of YAC transgene rescued *Hdh* nullizygous mice. Semi-thin sections of testes stained with toluidine blue from 8-month-old mice reveal the gross testicular morphology of mice with the YAC18 (a,b,c), YAC46 (d,e,f), and YAC72 (g,h,i) htt transgenes and either 100% of endogenous htt levels (+/+), 50% of endogenous htt levels (+/-) or absence of endogenous htt (-/-). Massive degeneration of spermatogenic cells occurs in the seminiferous tubules of mice expressing mutant htt with 46 or 72 polyglutamine repeats (panels i and f). The cell death is most pronounced in YAC72 (i), intermediate in YAC46 (f), and not present in YAC18 (c) rescued *Hdh* nullizygous mice. The human *HD* transgene in each of these lines of mice is identical except for the length of the CAG repeat, and these results suggest that this novel cell death phenotype is CAG repeat length-dependent. Increasing levels of endogenous htt markedly reduced the amount of spermatogenic cell degeneration (panels d,e and g,h) observed in YAC46 and YAC72 mice. (Bar = 100μm.)

Figure 13 shows morphologic, biochemical, and ultrastructural evidence for apoptotic cell death in the testes of YAC72 mice lacking endogenous htt. Massive death of spermatogenic cells was observed in YAC72 mice lacking endogenous htt by toluidine blue staining (A) which revealed decreased numbers of spermatogenic cells, and a disordered epiythelium filled with vacuoles compared to the well large numbers of spermatocytes in well ordered stratified epithelium of YAC72+/+ mice (C). Increased apoptosis was evident in the testes of YAC72 -/- mice by increased TUNEL labelling of spermatogenic cells (arrows in panel B) compared to YAC72 mice with normal levels of endogenous huntingtin (D). EM analysis of degenerating testicular cells from YAC72 (-/-

) mice also provided evidence of apoptosis. Ultrastructural analysis of testes from YAC72 mice nullizygous for *Hdh* reveals massive cell death of spermatids, phagocytosis of degenerating cells, and formation of multinucleated giant cells. The epithelium of YAC72 mice lacking endogenous htt was characterized by degenerating spermatids filled with cytoplasmic vacuoles (E), phagosomes containing shrunken electron-dense spermatids engulfed within Sertoli cells (F), and spermatogenic giant cells (G). Bars (A-D) = 100 μm, (E) = 10 μm, (F) = 5 μm, (G) 10 μm.

Figure 14 shows protein aggregates in YAC72 (-/-) mice. Ultrastructural analysis of the testes of YAC72 mice lacking endogenous htt revealed the occasional presence of abnormal aggregates of intracellular protein (arrows) within elongate spermatids (A), sertoli cells (B), and sperm tails (C). The composition of these protein aggregates is not entirely clear, but they resemble the ultrastructural appearance of huntingtin aggregates found in human HD brain tissue. Ectopic microtubule bundles (D) and manchettes (E) were also identified (asterisks). The bundle in E is in a spermatogonium. N = nucleus. Bars (A, B) = 5 μm, (C,D) = 1 μm, (E) = 5 μm.

Figure 15 presents immunocytochemical analysis of protein aggregates and actin distribution in sections from the testes of YAC72 -/- mice. Abnormal protein aggregates within degenerating spermatogenic cells in the testes of YAC72-/- mice contain huntingtin (Figures A, phase, and B, immunofluorescence). In normal epithelium (Figures E, phase, and F, fluorescence), actin filaments in Sertoli cells are concentrated in unique adhesion plaques (ectoplasmic specializations) that occur at apical sites of attachment to spermatids (Apical) and at basal sites of attachment to neighbouring Sertoli cells (Basal). In YAC72-/- mice (Figs. G-J), filament bundles (asterisks) in apical regions occur in areas not associated with spermatid heads, although filament bundles at basal sites occur in their normal position. Bars (A-D) = 10 μm, (E-J) = 50 μm.

Figure 16 shows the effect of htt over-expression on body weight. Transgenic mice aged 5-8 months old that over-express wild-type htt (n = 11) had a significantly increased total body mass compared to sex and age matched control mice (n = 12). The average body weight was approximately 25% greater for the transgenic mice compared to wild-type.

The increased body weight of the transgenic mice suggests that the wild-type htt is causing increased cellular survival or proliferation in general in these mice.

Figure 17 shows the effect of transfecting NIH3T3 cells with a wild-type huntingtin gene or a known oncogene (*ras*). Cellular proliferation was assessed following transient transfections of NIH3T3 cells using a standardised colorometric assay of cell number. Transfection of a full-length htt construct or the oncogene ras each lead to a significant increase in cellular proliferation of approximately 3-fold compared to empty vector alone.

Table 1 demonstrates the outcome of crosses between YAC transgenic mice that are heterozygous for the *Hdh* null allele (huntingtin knockout mice). The total number of live-born offspring is given for each transgenic line. Note that the offspring have the expected 1:2:1 ratio indicating that there was no significant foetal loss and that the human transgene is capable of rescuing the *Hdh* nullizygous state.

DETAILED DESCRIPTION OF THE INVENTION

Cellular contents and cells themselves are constantly in a state of flux, known as turnover. Growth and proliferation of cells is intimately associated with cell death, for example, as a result of apoptosis or necrosis. The state of cells can vary between excess proliferation, normal proliferation, steady state, cell death and abnormal cell death (Figure 1). The end result of disease or disorders oftentimes results from in an imbalance between the generative aspects of the lifecycle and the destructive aspects and the consequent abnormal proliferation of cells or abnormal cell death.

Thus, cancer can be seen to result from cellular growth being out of proportion from cellular death, resulting in rapid proliferation of cellular material. On the other hand, cellular death out of proportion from regeneration can result in the destruction of crucial areas of tissue, as observed in degenerative diseases such as, but not limited to, Parkinson's Disease, Amyotrophic Lateral Sclerosis, Alzheimer's disease or Huntington's Disease.

At the most general level, this invention provides huntingtin protein, biologically active fragments of the huntingtin protein, nucleic acid sequences encoding such proteins, and

antagonists to Huntingtin protein, and their use to modulate cell survival. In particular, these components are used to either activate or attenuate the apoptotic pathway within cellular material, in order to facilitate the treatment of conditions where there is a dysregulation of cell death (e.g. apoptosis) or cellular proliferation. Thus, the therapeutic application pertains to diseases and disorders such as Huntington disease, neurodegenerative diseases, stroke, etc, where there is a need to attenuate apoptosis, in addition to diseases such as cancer where there is a need for decreased cellular proliferation.

Definitions

The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook *et al.* Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) which are provided throughout this document. Standard techniques are used for chemical syntheses, chemical analyses, and biological assays.

As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

"Huntingtin" refers to the huntingtin protein, the mutant form of which is associated with Huntington disease.

"Naturally occurring" as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring.

"Polypeptide fragment" refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is usually identical to the corresponding positions in the naturally occurring sequence deduced, for example, from a full-length cDNA sequence.

"C terminus" means the half of the wild-type huntingtin protein that includes the C-terminus. The term is used synonymously with C-terminal fragment or C-terminal domain.

"N terminus" means the half of the wild-type huntingtin protein that includes the N-terminus.

"Antagonist" as used herein means any molecule that is capable of interacting with huntingtin protein in such a way that it interferes with the normal pro-survival function of the protein. Examples of antagonists according to the present invention are antisense oligonucleotides and anti-huntingtin antibodies.

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco, incorporated herein by reference).

The Use of Huntingtin Protein and Biologically Active Fragments Thereof

The present invention provides for the use of Huntingtin protein to attenuate cell death, thereby promoting cell survival, in the treatment of diseases or disorders wherein there is an imbalance between the generative and degenerative aspects of the cell, thus resulting in inappropriate cell death; such as, Alzheimer's disease, amytrophic lateral sclerosis, Huntingtin's disease, Parkinson's disease and retinal degeneration.

The huntingtin protein is expressed throughout the body, suggesting that taking advantage of the pro-survival properties of huntingtin, or biologically active fragments thereof, can be used in therapies where there is chronic excessive cell death (e.g. HD, Alzheimer's disease) or acute cell death (stroke and spinal cord injury).

The polyglutamine group of neurodegenerative diseases, including the spinocerebellar ataxias, HD, spinobulbar muscular atrophy (SBMA) and dentatorubralpallidoluysian atrophy (DRPLA), has been proposed to have similar molecular pathogeneses. For example, apoptotic cell death, caspase activation and protein deposition are common

events in the degenerative processes of these disorders. Therefore, the pro-survival effect of huntingtin, or biologically active fragments thereof, can act as a general therapeutic for the entire group of polyglutamine diseases.

Additionally, aberrant aggregation is a common feature of many neurodegenerative diseases, including the polyglutamine diseases (intracellular inclusions), Alzheimer's disease (amyloid plaques) and Parkinson's disease (Lewy bodies). The formation of protein aggregates may be involved at some stage in disease pathogenesis. Huntingtin was shown to reduce aggregation of mutant huntingtin *in vitro*, suggesting a potential therapeutic role of the C-terminus in preventing "aggregation disease" in general.

Finally, recent work has shown the involvement of huntingtin in haematopoiesis (Metzler *et al.* (2000) *Hum Mol Genet* 9, 387-94), thus, huntingtin has potential therapeutic value in treatment of diseases with aberrant blood cell production and maturation.

In the absence of wild-type huntingtin, the expression of mutant huntingtin in male HD mice results in infertility, testicular atrophy and apoptosis. Increasing the levels of endogenous wild-type huntingtin protects against the testicular phenotype. The results provided in Figures 2 – 4, 14 demonstrate that testicular cell death is CAG repeat size dependent and is modulated by the level of endogenous huntingtin. In particular, Figure 2 clearly indicates that addition of exogenous normal huntingtin, with only 18 CAG repeats, does not cause cell death, whereas the addition of exogenous huntingtin with 46 or 72 CAG repeats results in increasing levels of cell death. In humans, 18 CAG repeats in the huntingtin protein does not result in disease formation, whereas both 46 and 72 repeats do cause HD in humans. Further, Figures 3, 4 and 14 show that increased apoptosis and protein aggregation can be attributed to the presence of huntingtin with 72 CAG repeats.

According to another embodiment of the present invention biologically active fragments of huntingtin protein can be used to attenuate the cellular pathway of apoptosis. These fragments can arise from any portion of the huntingtin protein. In one embodiment, such fragments arise from the C-terminus of the protein which further demonstrates that huntingtin is able to modulate apoptosis. It is not necessary, however, that the actual C-terminus be included in such fragments. Rather this portion of the protein provides a good

source of candidate biologically active fragments that can then be further tested for activity.

The data presented in Figures 5 – 8 demonstrates the effectiveness of biologically active fragments derived from the C-terminus of Huntingtin protein. Sequence analysis of the C-terminus has not identified any similarities to other proteins that would indicate its function. The observation of anti-apoptotic and anti-aggregation properties was a surprising result obtained from functional studies (described below) using a C-terminal fragment of the huntingtin protein.

One embodiment of the present invention is, therefore, the protection of cells from cell death as a result of the function of huntingtin protein and/or biologically active fragments derived from any portion of huntingtin.

Another embodiment is the reduction of aggregation as a result of the anti-aggregation activity, or function, of huntingtin protein and/or biologically active fragments derived from any portion of huntingtin.

Another embodiment of the present invention takes advantage of the activities of the biologically active fragments derived from huntingtin to protect mammals with HD from testicular degeneration and cell death. This has been demonstrated in male mice that are models for HD. Thus, the pro-survival activities of huntingtin, as outlined below, bring about the therapeutic effects that have been observed in male mice models for HD. This demonstrates that biologically active fragments derived from huntingtin act to regulate cell death, thereby improving cell survival and prognostic outcome of the condition being treated.

The pro-survival activity of biologically active fragments derived from huntingtin fits well with what is known about huntingtin, HIP-1 and caspase activation. It has been shown, in an *in vitro* HD model, that cleavage of huntingtin by caspase is necessary for cell death to occur. The resulting N-terminal polyglutamine-containing fragment of huntingtin has been shown to be toxic to cells. Furthermore, HIP-1 is a protein that interacts tightly with normal huntingtin but only weakly with mutant huntingtin. HIP-1 induces cell death by an

apoptotic mechanism, and the amount of cell death is increased in the presence of mutant huntingtin.

In one embodiment of the present invention there is provided a therapeutic means based on delivery of biologically active fragments derived from a domain of huntingtin, such as, but not limited to, the C-terminal domain. The ability of a fragment derived from the C-terminus to reduce HIP-1 mediated toxicity is demonstrated from neuronal cell culture studies in which the expression of huntingtin significantly reduced toxicity due to HIP-1 (Figure 7). Therefore, if HD is partially or wholly due to HIP-1 toxicity, then delivery of peptides derived from huntingtin, or nucleic acid sequences encoding such protein, to susceptible cells will have a therapeutic benefit.

The ability of peptides derived from huntingtin to reduce polyglutamine-mediated toxicity has also been demonstrated in neuronal cell culture studies. For example, the expression of huntingtin was demonstrated to protect against cell death due to the presence of mutant huntingtin (Figure 6).

An advantage of utilising biologically active fragments of huntingtin, such as, but not limited to, those arising from the C-terminus of huntingtin, as a therapeutic approach to the treatment of huntingtin and other polyglutamine disease, over alternative pharmaceutical-based therapies, lies in the specific targeting of part of the pathological pathway. This is an improvement over therapies aimed at the symptoms of the disease rather than at the cause.

Candidate fragments are selected from random fragments generated from the wild-type huntingtin. In one embodiment of the present invention the fragments are generated from the C-terminal domain of the wild-type huntingtin. Methods for generating the candidate polypeptide fragments are well known to workers skilled in the art and include enzymatic, chemical or mechanical cleavage of the native protein, expression of nucleic acids encoding such fragments, etc.

Assays to Determine the Pro-Survival Activity of a Candidate Molecule

Screening for the pro-survival effect of the candidate huntingtin protein fragment *in vitro* can be performed using cell lines transfected with the gene encoding the candidate fragment. The transfected cells are treated with a pro-apoptotic drug and one of several easily defined cellular markers of viability is measured. The markers used include, but are not limited to, morphological features of apoptosis, caspase activity and mitochondrial function. Additionally, cell lines can be created that stably express a biologically active fragment of huntingtin that can be used as a reagent for screening the effectiveness of the fragment in protecting against multiple pro-cell death stimuli.

The pro-survival effect of the huntingtin protein can be measured *in vitro*, for example, by transfecting cell lines with huntingtin, treating with a pro-cell death drug and assaying for several cellular markers. These markers include: (1) cell death, measured by cell morphology; (2) mitochondrial viability, measured by enzyme activity; and (3) aggregate formation, measured by immunofluorescence staining. *In vivo* effects of huntingtin in the transgenic mice can be assessed by: (1) observation of testicular cellular morphology by microscopy; and (2) DNA fragmentation using TUNEL staining.

1) Cell death assays.

In one example, human neuronal precursor cell line NT2 cells are transfected with huntingtin or control cDNAs using lipofectamine, according to the manufacturer's directions. Cell death can be quantified in NT2 cells by co-transfection of the expression constructs with a plasmid containing the *LacZ* gene at a 4:1 ratio, and the cells are stained for β-galactosidase activity at 24 hr post-transfection using standard procedures. The pro-survival effect of the candidate protein is assessed by incubating transfected cells with the pro-apoptotic drug tamoxifen at various concentrations. An apoptotic morphology is scored as blue-staining cells that are rounded up, blebbed and condensed, which are clearly distinguished from viable cells that are flat and have neurite-like extensions.

2) Mitochondrial viability.

In one example, HEK 293T cells are seeded in 96-well plates and transfected with huntingtin or *LacZ* DNA using a standard calcium phosphate protocol as described (Hackam et al., 1998). Transfected cells in 96-well plates are treated at 48 hours post-

transfection with a various concentrations of tamoxifen. Cell viability is measured by MTT assay at 24 hr post-transfection by incubating the cells for 2 hr in a 1:10 dilution of WST-1 reagent (Boehringer Mannheim) and release of formazan from mitochondria is quantified at 450 nm using an ELISA plate reader. Mock transfected, vector only and *LacZ* transfected cells serve as controls for transfection-related toxicity. One way ANOVA and Newman-Keuls test are used for statistical analysis. Statistical analyses of the cell death data in NT2 and HEK cells are performed using one-way ANOVA and Newman-Keuls post-comparison tests.

3) Aggregate formation.

In one example, human embryonic kidney cells (HEK 293T) are transfected and processed for immunofluorescence by growing cells on glass coverslips and transfecting using a standard calcium phosphate protocol. At 48 h post-transfection, the cells are treated with tamoxifen to induce aggregate formation, then processed for immunofluorescence. The cells are fixed, permeabilised, then incubated with anti-huntingtin antibody. Secondary antibodies conjugated to a marker, such as FITC with the use of DAPI (4',6'-diamindino-2-phenylindole) as a nuclear counter-stain. Appropriate control experiments are performed to determine the specificity of the antibodies, including secondary antibody only and mock transfected cells. The cells are viewed with microscope, digitally captured with a CCD camera and the images are colourised and overlapped. The proportion of cells with aggregates is presented as a percent of the total number of cells expressing huntingtin.

In vivo effects of huntingtin in the transgenic mice are assessed by, for example: (1) observation of breeding efficiency; (2) analysis of testicular cellular morphology by microscopy and DNA fragmentation using TUNEL staining.

1) Generation of experimental mice and assessment of breeding efficiency.

In one example, heterozygous knockout (KO) mice are bred with YAC transgenic mice to generate a series of F1 generation mice that express the YAC transgene on a background that is heterozygous for endogenous huntingtin (one copy of the KO allele). These F1 generation mice are then bred to generate the experimental F2 mice with the following genotypes: YAC transgene positive or negative on a background of normal endogenous htt (+/+), half normal endogenous htt (+/-), and lacking endogenous htt (-/-). Genotyping is

performed by standard PCR based techniques on genomic DNA from tail clippings prepared by phenol-chloroform extraction. Protein expression is determined by Western blot, using an anti-huntingtin antibody to probe the blot.

Mice of each genotype are set-up with FVB/NJ wild-type mates and allowed to remain in breeding cages for a minimum of 4 months with the number of pregnancies, litters, and pups recorded. Several breeding pairs are set-up per genotype and the results of a minimum of 20 months of combined breeding time tabulated per genotype.

2) Analysis of testicular morphology.

In one example, whole testes are removed from adult mice of each genotype and one testicle prepared for immunocytochemistry and TUNEL staining and one for semi-thin sections stained with toluidine blue and electron microscopy. For immunocytochemical and TUNEL analyses the testes are immersion-fixed overnight in paraformaldehyde, cyropreserved in sucrose solution, frozen, and cryostat sectioned. For immunocytochemistry, slide mounted sections are incubated in blocking solution for one hour and then in diluted primary antibody solution overnight. After serial washes in phosphate buffered saline (PBS), the sections are incubated in diluted secondary antibody, washed, and mounted under coverslips. TUNEL staining is performed on similar sections using techniques known in the art, for example by using the *In situ* cell death detection kit (Boehringer Mannheim), according to manufacturer's instructions. For semi-thin toluidine blue staining and EM analysis, testes are cut in 40 μm coronal sections using a vibratome collected in PBS, osmicated (1% OsO_4 in 0.1M cacodylate buffer), rinsed, and stained overnight in 2% aqueous uranyl acetate. All the sections used are dehydrated in ascending concentrations of ethanol and propylene oxide (1:1) and flat embedded in Eponate 12. Semi-thin sections (1.5 μm) are cut using a Leica Ultracut S ultramicrotome, counterstained with toluidine blue or cresyl violet, differentiated in 95% alcohol and coverslipped. Sections are visualised using a Nikon Microphot FXA equipped with a 60x oil immersion lens. Ultrathin sections (90 nm) are cut using a Leica Ultracut S ultramicrotome, counterstained with 5% aqueous uranyl acetate for 5 minutes followed by lead citrate for 5 minutes. Thin sections are examined using an electron microscope.

Antagonists of Huntingtin Protein

One embodiment of the present invention provides antagonists of huntingtin protein that decrease the pro-survival function of the protein and thereby reduce abnormal cell proliferation. A related embodiment of the present invention is the use of such antagonists in the treatment of diseases or disorders having a dysregulation of cellular proliferation. In these diseases and disorders cell proliferation is out of balance with cell death, which results in inappropriate cell growth. Cancer is one example of a disease characterised by excess cell proliferation.

A huntingtin antagonist according to the present invention can be an antibody that binds to the Huntingtin protein and effectively decreases or eliminates the pro-survival activity of said protein. Alternatively, an antagonist may be a biologically inactive form, or fragment, of huntingtin protein that interferes with the action of the wild-type protein; for example, dominant negative mutants of huntingtin protein.

In a related embodiment of the present invention the huntingtin antagonist is an antisense oligonucleotide that targets the cellular gene (or mRNA transcribed from the gene) that encodes huntingtin protein. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that modulation of expression of the protein will result. Within the context of the present invention, an exemplary intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. In the context of the present invention, "translation initiation codon" refers to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding huntingtin protein, regardless of the sequence(s) of such codons.

The term "translation initiation codon region" refers to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the term "translation termination codon region" refers to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the

translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N^7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also targets. It has also been found that introns can also be effective target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridise sufficiently well and with sufficient specificity, to give the desired effect of decreasing the pro-survival activity of huntingtin protein within the cell.

"Specifically hybridisable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to

that of its target nucleic acid to be specifically hybridisable. An antisense compound is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

In the context of the present invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides can exhibit desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. One embodiment of the present invention provides antisense oligonuceotides comprising from about 8 to about 50 nucleotides. In a related embodiment the antisense oligonucleotides comprise from about 15 to about 30 nucleotides.

An alternative embodiment of the present invention provides huntingtin antagonists that are small molecules which bind to huntingtin protein thereby interfering with the normal pro-survival function of the protein. Examples of small molecules include, but are not limited to, small peptides or peptide-like molecules. Similarly, the present invention provides small molecule antagonists that bind to a nucleic acid encoding wild-type huntingtin and interfere with expression of the protein, thereby reducing the pro-survival effect of the protein.

Assay to Determine the Antagonist Activity of a Candidate Molecule

One embodiment of the present invention provides an assay for use in screening candidate molecules for antagonist activity against huntingtin function. This assay can be used to

screen candidate molecules for anti-proliferative functions, which can be used, for example, as anti-cancer therapeutics.

In one example of this assay NIH3T3 cells are first transfected with a wild-type huntingtin gene. The transfected cells show a significant increase in cellular proliferation (Figure 17). The candidate molecule is added to the cell culture before, during or after proliferation of the transfected cells *in vitro*. This includes, but is not limited to, conditions in which the cells are cultured in a state of contact inhibition, in soft agar or in an animal. In one embodiment of the present invention the cells are cultured in a mouse in order to demonstrate cell proliferation or tumour growth *in vivo*. A control assay is performed simultaneously in the absence of the candidate molecule. The degree of proliferation in the presence of the candidate molecule is compared to the degree of proliferation in the control assay, where a reduced degree of proliferation in comparison to the control is indicative of an antagonist activity.

In a related embodiment the present invention provides screening kits comprising components required for performing the above anti-proliferation screening assay. Such a kit comprises NIH 3T3 cells transfected with wild-type huntingtin and instructions for use.

Preparation of Proteins and Polypeptides of the Present Invention

The proteins and polypeptide of the present invention, including wild-type huntingtin, biologically active fragments thereof and proteinaceous antagonists of wild-type huntingtin, can be prepared from cell extracts or through the use of recombinant techniques. In general, the proteins and polypeptides according to this invention can be produced by transformation (transfection, transduction, or infection) of a host cell with all or part of a DNA encoding such a protein or polypeptide in a suitable expression vehicle. Suitable expression vehicles include: plasmids, viral particles, and phage. For insect cells, baculovirus expression vectors are suitable. The entire expression vehicle, or a part thereof, can be integrated into the host cell genome. In some circumstances, it is desirable to employ an inducible expression vector, e.g., the LACSWITCH™ Inducible Expression System (Stratagene, LaJolla, Calif.).

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The huntingtin protein can be produced in a prokaryotic host (e.g., *E. coli* or *B. subtilis*) or in a eukaryotic host (e.g., *Saccharomyces* or *Pichia*; mammalian cells, e.g., COS, NIH 3T3, CHO, BHK, 293, or HeLa cells; or insect cells). Proteins and polypeptides can also be produced using plant cells. For plant cells viral expression vectors (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors (e.g., Ti plasmid) are suitable. The methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel *et al.* (1994) Current Protocols in Molecular Biology, John Wiley & Sons, New York; and various expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (Pouwels *et al.*, 1985, Supp. 1987).

The host cells harbouring the expression vehicle can be cultured in conventional nutrient media adapted as need for activation of a chosen gene, repression of a chosen gene, selection of transformants, or amplification of a chosen gene.

Furthermore, the proteins and polypeptides of the present invention can be produced as fusion proteins. One use of such fusion proteins is to improve the purification or detection of the protein or polypeptide. For example, huntingtin or a fragment thereof, can be fused to an immunoglobulin Fc domain. Such a fusion protein can be readily purified using a protein A column.

Specific initiation signals may be required for efficient translation of cloned nucleic acid sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire wild-type huntingtin gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. In other cases, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both

natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators (Bittner *et al.* (1987) *Methods in Enzymol.* 153, 516).

In addition, as would be readily appreciated by a worker skilled in the art, a host cell may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.

Preparation of Compositions and Therapeutic Formulations

One embodiment of the present invention is the use of wild-type huntingtin, biologically active fragments of wild-type huntingtin, or antagonists of wild-type huntingtin protein in the preparation of pharmaceutical compositions, used for the treatment of conditions in which there is dysregulated cell death or cellular proliferation. Such pharmaceutical compositions comprise wild-type huntingtin, a biologically active fragment of wild-type huntingtin, a combination thereof, or one or more antagonists of wild-type huntingtin and a pharmaceutically acceptable diluent or excipient. A related embodiment is the compositions as above which additionally comprise a therapeutic compound, which may be chosen from the group comprising: antibiotics, anti-inflammatories, antidepressants, etc.

The pharmaceutical compositions of the present invention may be administered orally, topically, parenterally, by inhalation or spray or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques.

Methods of Delivery for the Compositions and Formulations

The huntingtin protein, biologically active fragments thereof, or antagonists of huntingtin protein may also be employed in accordance with the present invention by expression of such proteins *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding huntingtin or a biologically active fragment thereof.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding huntingtin, or a biologically active fragment thereof, may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering huntingtin, or a biologically active fragment thereof, by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus that may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

In addition, when the antagonist of huntingtin comprises an antisense oligonucleotide, it is also contemplated that said oligonucleotide may be administered without additional carrier or delivery molecules. Injection of "naked" DNA is well-known in the art as an effective method of administering antisense therapy (for example, see Felgner *et al.*, U.S. Patent No. 5,580,589).

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or

sale for human administration. In addition, the pharmaceutical compositions of the present invention may be employed in conjunction with other therapeutic compounds.

To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

Example 1: Determination of the *In Vivo* Effect of Reduced Normal Huntingtin

To determine the *in vivo* effect of reduced expression of normal huntingtin, mice were bred to be transgenic for human huntingtin and to express various levels of wild-type mouse huntingtin. The breeding strategy allowed the generation of mice expressing huntingtin with different CAG repeat sizes, with or without co-expression of normal huntingtin.

Heterozygous knockout (KO) mice that had only one copy of the wild-type huntingtin allele were bred with YAC transgenic mice to generate a series of F1 generation mice that express the YAC transgene on a background that is heterozygous for endogenous huntingtin ("YAC rescued mice"). The YAC transgenes used in these studies contained 18, 46 or 72 CAG repeats. The F1 generation mice were then bred to generate the experimental F2 mice with the following genotypes: YAC transgene positive or negative on a background of normal endogenous htt (+/+), half normal endogenous htt (+/-), and lacking endogenous htt (-/-). Genotyping was performed by standard PCR based techniques on genomic DNA from tail clippings prepared by phenol-chloroform extraction (Ausubel *et al.* (1994) Current Protocols in Molecular Biology, John Wiley & Sons, New York). Protein expression was determined by Western blot in which 200 µg of total protein from homogenised testes was probed with the anti-huntingtin antibody (HD3).

The cellular effect of the huntingtin expression was determined by analysing the testicular morphology of YAC rescued huntingtin knockout mice. Spermatocyte degeneration,

which was found to be CAG repeat size-dependent, was also shown to be modulated by the level of endogenous huntingtin (see Figure 2).

Whole testes were removed from 8 month old adult male mice of each genotype. For semi-thin toluidine blue staining, testes were cut in 40 μm coronal sections using a vibratome, collected in PBS, osmicated (1% OsO_4 in 0.1M cacodylate buffer), rinsed, and stained overnight in 2% aqueous uranyl acetate. All the sections used were dehydrated in ascending concentrations of ethanol and propylene oxide (1:1) and flat embedded in Eponate 12 Semi-thin sections (1.5 μm) were cut using a Leica Ultracut S ultramicrotome, counterstained with Toludine Blue or Cresyl Violet, differentiated in 95% alcohol and coverslipped. Sections were visualised using a Nikon Microphot FXA equipped with a 60x oil immersion lens.

Example 2: Quantitation of Increased Apoptosis with Mutant Huntingtin

TUNEL staining was used to quantitate apoptosis in the YAC rescued transgenic mice. These experiments demonstrated increased apoptosis in the testes of YAC72 rescued huntingtin knockout mice, indicating that absence of endogenous huntingtin lead to cell death (see Figure 14).

Whole testes were removed from adult mice of each genotype. For TUNEL analyses, the testes were immersion-fixed overnight in paraformaldehyde, cyropreserved in sucrose solution, frozen, and cryostat sectioned at 10 μm. For immunocytochemistry, slide mounted sections were incubated in blocking solution for one hour and then in diluted primary antibody solution overnight. After serial washes in phosphate buffered saline (PBS), the sections were incubated in diluted secondary antibody for two hours, washed, and mounted under coverslips. TUNEL labelling was performed using standard techniques on frozen sections of testes that were lightly immersion-fixed in 3% paraformaldehyde, using the *In situ* cell death detection kit (Boehringer Mannheim), according to the manufacturer's instructions.

Example 3: Demonstration of Abnormal Protein Aggregation

Mutant polyglutamine containing proteins are known to aggregate into large amorphous protein clumps. These aggregates are considered to be markers for pathological changes *in vivo*, and their frequency correlates with cell death in culture. In the YAC72 transgenic mice, huntingtin aggregates were observed in the striata late in the pathological process (Hodgson et al. *Neuron* 1999, 23:181-92). Ultrastructural analysis using electron microscopy was used to determine the effect of endogenous huntingtin on the formation of aggregates. The testes of YAC72 mice lacking endogenous huntingtin contained abnormal aggregates within spermatids, sertoli cells and sperm tails (see Figure 3).

For EM analysis, testes were cut in 40 μm coronal sections using a vibratome, collected in PBS, osmicated (1% OsO_4 in 0.1M cacodylate buffer), rinsed, and stained overnight in 2% aqueous uranyl acetate. All the sections used were dehydrated in ascending concentrations of ethanol and propylene oxide (1:1) and flat embedded in Ultrathin sections (90 nm) were cut using a Leica Ultracut S ultramicrotome, counterstained with 5% aqueous uranyl acetate for 5 minutes followed by lead citrate for 5 minutes. Thin sections were examined using a HITACHI H-7500 electron microscope.

Example 4: Characterisation of Degeneration Phenotype

Additional electron microscopic analysis was used to characterise the degeneration phenotype in the YAC72 mice lacking endogenous huntingtin (see Figure 4).

Ultrastructural analysis was performed as described in Example 3. The testes were shown to have elevated spermatid cell death, phagocytosis of degenerating cells and formation of multinucleated giant cells. These data demonstrate the role of endogenous huntingtin in protecting against mutant huntingtin-induced degeneration.

Example 5: Demonstration of the Pro-Survival and Anti-Apoptotic Effect of Huntingtin

Tamoxifen-Induced Cell Death

Expression of the C-terminus of the huntingtin protein was shown to be protective against tamoxifen-induced cell death in transfected cell lines (see Figure 5). Tamoxifen is a cell permeable compound that leads to caspase activation and cell death. The survival effect of huntingtin was measured *in vitro* by transfecting cell lines with huntingtin, treating with a pro-apoptotic drug and assaying for mitochondrial viability using the MTT assay. In the MTT assay, a reduction in mitochondrial viability is indicative of cell death.

For the viability assays, HEK 293T cells were seeded at a density of 5×10^4 cells into 96-well plates and transfected with 0.1 μg huntingtin or LacZ DNA using a standard calcium phosphate protocol as described (Hackam *et al.* (1998) *J Cell Biol.* **141**: 1097-105). Transfected cells in 96-well plates were treated at 48 hours post-transfection with a various concentrations of tamoxifen for 4 hr. Cell viability was measured, at 24 hr post-transfection, by incubating the cells for 2 hr in a 1:10 dilution of WST-1 reagent (Boehringer Mannheim) and release of formazan from mitochondria was quantified by the MTT assay at 450 nm using an ELISA plate reader (Dynatech Laboratories). Mock transfected, vector only and LacZ transfected cells served as controls for transfection-related toxicity. Statistical analyses of the cell death data were performed using one-way ANOVA and Newman-Keuls post-comparison tests.

Mutant Huntingtin-Induced Cell Death

An additional cell line was tested with a different cell death induction paradigm, to demonstrate that the C-terminus protection is not specific to tamoxifen-induced cell death. We show that the C-terminus of huntingtin confers protection against mutant huntingtin-induced toxicity in NT2 cells (see Figure 6). Furthermore, this data supports the *in vivo* evidence that wild-type huntingtin is essential for protecting against mutant huntingtin in the YAC72 mice.

Human neuronal precursor cell line NT2 cells were co-transfected at 40% density with mutant huntingtin along with the C-terminus or control pyruvate kinase cDNAs, using

lipofectamine (GibcoBRL), according to the manufacturer's directions. Cell death was quantified in NT2 cells by co-transfection of the expression constructs with a plasmid containing the *LacZ* gene at a 4:1 ratio, and the cells were stained for β-galactosidase activity at 24 hr post-transfection using standard procedures. The survival effect of the huntingtin protein was assessed by incubating transfected cells with the pro-apoptotic drug tamoxifen for 4 hrs at various concentrations. An apoptotic morphology was scored as blue-staining cells that were rounded up, blebbed and condensed, which were clearly distinguished from viable cells that were flat and had neurite-like extensions. The cell death data was analysed for statistical significance using one-way ANOVA and Newman-Keuls post-comparison tests.

HIP-1-Induced Cell Death

The huntingtin interacting protein HIP-1 is a pro-apoptotic protein that rapidly induces cell death. HIP-1 induces cell death in a caspase-dependent manner, and toxicity is exacerbated in the presence of mutant huntingtin, suggesting that HIP-1 may be involved in HD pathogenesis. We used HIP-1-induced toxicity to assess whether the C-terminus of huntingtin confers protection against a toxic protein-mediated cell death, in addition to its effect on tamoxifen-induced death (see Figure 7).

NT2 cells were co-transfected with mutant HIP-1 and the C-terminus, or HIP-1 and the control pyruvate kinase cDNA, using lipofectamine (GibcoBRL). Cell death was measured by morphological changes using co-transfection of a plasmid containing the *LacZ* gene, as described above. We demonstrated that expression of the C-terminus in NT2 cells reduced cell death induced by HIP-1. These data indicate that the protective activity of the C-terminus may have a functional role in HIP-1-mediated cell death *in vivo*.

Example 6: Reduction of Mutant Huntingtin Aggregation by C-Terminus of Huntingtin

Mutant huntingtin protein forms aggregates in HD brains, transgenic mice and in cell culture. We have previously shown that truncated and full-length huntingtin containing 128 CAG repeats readily forms aggregates in HEK 293T cells when the cells are exposed to apoptotic stress by tamoxifen (Hackam *et al.* (1998) *J Cell Biol.* 141:1097-105). Therefore, huntingtin aggregates can be used as an additional marker of cell viability. To

determine whether the C-terminus of huntingtin has an effect on this pathological marker of HD, we cotransfected the C-terminus with mutant huntingtin and induced aggregate formation with tamoxifen or HIP-1 expression (see Figure 8).

Human embryonic kidney cells (HEK 293T) were transfected with truncated mutant huntingtin with 128 CAG repeats and processed for immunofluorescence by growing cells on glass coverslips and transfecting at 30% confluency using a standard calcium phosphate protocol. At 48 h post-transfection, the cells were treated with 35 μM tamoxifen (Sigma) for 1 hour to induce aggregate formation, then processed for immunofluorescence. The cells were fixed in 4% paraformaldehyde/PBS, permeabilised in 0.5% Triton X-100/PBS for 5 min, then incubated with anti-huntingtin antibody MAB2166 (Chemicon) (1:2500 dilution) diluted in 0.4% BSA/PBS. Secondary antibodies conjugated to FITC (Jackson Laboratories) were used at 1:600-1:800 dilutions, and DAPI (4',6'-diamindino-2-phenylindole, Sigma) was used as a nuclear counter-stain. Appropriate control experiments were performed to determine the specificity of the antibodies, including secondary antibody only and mock transfected cells. The cells were viewed with a Zeiss (Axioscope) microscope, digitally captured with a CCD camera (Princeton Instrument Inc.) and the images were colourised and overlapped using the Eclipse (Empix Imaging Inc.) software program. The proportion of cells with aggregates is presented as a percent of the total number of cells expressing huntingtin. These data indicate that the C-terminus of huntingtin protein is able to reduce mutant huntingtin protein aggregate formation in transfected cells.

Example 7: Offspring from Crosses of YAC Transgenic Mice

In Table 1 shows the offspring generated by crosses between YAC transgenic mice that are heterozygous for the huntingtin null allele (huntingtin knockout mice). These data demonstrate that there was no observed perinatal loss, and that the human transgene is capable of rescuing the huntingtin nullizygous state, indicating that the transgene has normal huntingtin developmental expression and function.

Mice of each genotype were set-up with FVB/NJ wild-type mates and allowed to remain in breeding cages for a minimum of 4 months with the number of pregnancies, litters, and

pups recorded. Several breeding pairs were set-up per genotype and the results of a minimum of 20 months of combined breeding time tabulated per genotype.

EXAMPLE 8: Demonstration That Over-Expression Of Full-Length Wild-Type Huntingtin In Mice Confers Protection Against Excitotoxic Neurodegeneration

Yeast artificial chromosome (YAC) transgenic mice were generated that over-express wild-type human huntingtin (line 212) at 2-3 times the levels of endogenous htt in wild-type mice (FVB/NJ) (Hodgson *et al.*(1996) *Hum. Mol. Genet.* 5, 1875-1885). Fifteen of these transgenic and 23 control FVB/NJ mice 4-8 months of age each received a single intraperitoneal injection of 25 mg/kg kainic acid (KA) or an equal volume of vehicle (PBS). Intraperitoneal injections of kainic acid injections cause prolonged seizures in mice, and each mouse was observed continuously for two hours following injection. The occurrence, severity, and duration of seizures was recorded.

One week following KA injection, brains were removed from one group of the mice and immediately frozen in isopentene on dry ice. Serial 30 µm coronal cryostat sections were cut through the entire hippocampus. After every fifth section, two sections were removed for quantitative analysis and fixed in 3% paraformaldehyde for 30 minutes in preparation for histochemical staining. Degenerating neurons were identified in hippocampal sections by Fluoro-Jade™ histochemistry (Histo-Chem inc.), silver staining (FD Neurotechnologies) and by TUNEL™ labeling (Boehringer Mannheim). Fluoro-Jade™ is a fluorescent stain that labels degenerating neurons in fixed brain sections. The total number of degenerated hippocampal neurons labeled with Fluoro-Jade™ was recorded from the CA1, CA3, and total hippocampus regions of each section selected for quantitative analysis. Slide-mounted sections were viewed with a Zeiss (Axiovert) fluorescent microscope, digital photomicrographs were captured with a cooled CCD camera (Princeton), and degenerating hippocampal neurons manually counted in a blinded fashion. To assess the role of caspase activation in this process, the hippocampus and cerebellum were removed from a second group of animals 8 hours following KA- induced seizures. Caspase activity was measured in homogenized hippocampal and cerebellar samples using the fluorogenic substrate acetylated DEVD aminofluorocoumarin. DEVD-ase activity was standardized to protein content as determined by standard Lowry analysis.

Following KA-induced seizures YAC transgenic mice expressing 2-3 times the endogenous levels of wild-type huntingtin averaged approximately 50-fold less (81 vs. 4362, *** $p<0.0001$) degenerating hippocampal neurons than control animals (Figure 9a). Fluoro-jade brightly labeled the soma and large processes of degenerating hippocampal neurons within CA1 and CA3 of KA-treated transgenic and wild-type mice (Figure 9b, 9c and 9d). Degenerating neurons were predominantly restricted to the CA1 and CA3 regions of hippocampus. KA-induced neurodegeneration was significantly reduced in both the CA1 (3 vs. 2360, * $P<0.000001$) and CA3 regions (79 vs. 2003, ** $P< 0.0003$) of transgenic relative to control mice. Intraperitoneal injection of 25 mg/kg of kainic acid was sufficient to cause prolonged seizures in all the mice in this study irrespective of genotype. No seizure activity or neurodegeneration was observed in any mouse following injection of PBS.

Argyrophyllic labelling of neuronal soma was dramatically reduced in adjacent silver-stained hippocampal sections from transgenic (Figure 9e) compared to wild-type mice (Figure 9f. and 9g.) following KA-induced seizures, confirming the results of Fluoro-Jade™ staining using this well-established marker for neurodegeneration. TUNEL™ staining (Figure 9h.) labelled few apoptotic hippocampal neurons following KA-induced seizures in brains from transgenic mice, but many TUNEL™ labelled neurons were identified in wild-type brains (Figure 9i and 9j). Significantly less hippocampal caspase activation was evident in transgenic mice compared to wild-type mice following KA-induced seizures (Fig. 10, 14.5 +/- 1.49 vs. 20.8 +/- 1.77, * $P= 0.02$) in parallel to the observed effects of wild-type huntingtin on neurodegeneration. No significant difference was found in cerebellar caspase activation for transgenic vs. wild-type (Figure 10, 13.66 +/- 2.32 and 8.68 +/- 1.67). Degeneration does not occur in the cerebellum following KA-induced seizures.

Transgenic mice expressing 2-3 times endogenous levels of wild-type huntingtin were resistant to apoptotic neurodegeneration, having approximately 50-fold less kainic acid-induced hippocampal neurodegeneration than littermate controls. Caspase activity levels within the hippocampus were increased following KA-induced seizures, and there was less DEVD-ase activation in the presence of increased levels of wild-type huntingtin. These

data demonstrate a significant anti-apoptotic role for wild-type huntingtin in neurons of the central nervous system. This anti-apoptotic effect of wild-type huntingtin acts upstream of caspase activation. The DEVD-ase fluorogenic assay captures the enzymatic activity of caspase-2, -3, and –7, and does not identify the specific caspases activated in KA-induced seizures. Modulation of caspase-dependent pathways by wild-type huntingtin may alter the sensitivity of neurons to excitotoxic stress.

These results demonstrate that there is a relationship between normal huntingtin function and neuronal susceptibility to excitotoxicity. Increasing wild-type huntingtin expression levels either by drugs or using gene therapy has a therapeutic benefit as an anti-apoptotic agent for HD, and other neurodegenerative disorders.

EXAMPLE 9: Demonstration that Wild-Type Huntingtin Reduces the Cellular Toxicity of Mutant Huntingtin *In Vivo*

The mutation in Huntington disease (HD) is the expansion of a CAG trinucleotide repeat in the first exon of the *HD* gene (Huntington Disease Collaborative Research Group, 1993). Alleles containing expansions of greater than 35 CAG repeats are associated with the clinical phenotype of HD, with an earlier age of onset occurring with higher CAG repeat sizes (Andrew *et al.* (1993) *Nat. Genet.* 4, 398-403). The mutation in the *HD* gene produces a protein, huntingtin (htt), with an expanded polyglutamine tract. Proteolytic cleavage of huntingtin, possibly by caspases, produces N-terminal huntingtin fragments containing the expanded polyglutamine tract (Goldberg *et al.* (1996) *Nat. Genet.* 13, 442-449; Wellington *et al.* (1998) *J. Biol. Chem.* 273, 9158-9167; Wellington *et al.* (2000) *J. Biol. Chem.* 275, 19831-19838). N-terminal fragments of mutant expanded huntingtin have altered cellular interactions (Li *et al.* (1995) *Nat. Genet.* 25, 385-389; Burke *et al.* (1996) *Nat. Med.* 2, 347-349; Bao *et al.* (1996) *Proc. Nat. Acad. Sci. USA* 93, 5037-5042; Wanker *et al.* (1996) *Hum. Mol. Genet.* 6, 487-495; Kalchman *et al.* (1996) *J. Biol. Chem.* 271, 19385-19394; Kalchman *et al.* (1997) *Nat. Genet.* 16, 44-53), nuclear localisation (Davies *et al.* (1997) *Cell* 90, 537-548; DiFiglia *et al.* (1997) *Science* 277, 1990-1993; Becher *et al.* (1998) *Neurobiol. Dis.* 4, 387-397; Hackam *et al.* (1998) *Cell. Biol.* 141, 1097-1105; Schilling *et al.* (1999) *Hum. Mol. Genet.* 8, 397-407; Hodgson *et al.* (1999) *Neuron* 23, 181-192; Gutekunst *et al.* (1999) *J. Neurosci.* 19, 2522-2534; Wheeler *et al.*

(2000) *Hum. Mol. Genet.* 9, 503-513; Li *et al.* (2000) *Nat. Genet.* 25, 385-389), and are directly toxic to neuronal cells in a variety of *in vitro* model systems (Martindale *et al.* (1998) *Nat. Genet.* 18, 150-154; Sandou *et al.* (1998) *Cell* 95, 55-66; Hackam *et al.* (1998) *Cell. Biol.* 141, 1097-1105). These huntingtin fragments are also prone to intracellular aggregation and inclusion formation (Hackam *et al.* (1998) *Cell. Biol.* 141, 1097-1105; Martindale *et al.* (1998) *Nat. Genet.* 18, 150-154; Cooper *et al.* (1998) *Hum. Mol. Genet.* 7, 783-790), although the relevance of huntingtin aggregation to the pathogenesis of HD remains unclear (reviewed in Sisoda (1998) *Cell* 95,1-4). The expansion of polyglutamine residues in htt has been proposed to result in a novel toxic gain of function of the mutant protein (MacDonald and Gusella (1996) *Curr. Opin. Neurobiol.* 6, 638-643), but alterations in normal endogenous huntingtin levels and loss of htt function may also play a role in the pathogenesis of HD.

Huntingtin is a large protein of uncertain function that is ubiquitously expressed in many tissues of the body, but which has the highest levels in brain and testis (Sharp and Ross, (1996) *Neurobiol. Dis.* 3, 3-15). Mice homozygous for targeted disruption of *Hdh* (-/-), the murine homologue of the HD gene, die at embryonic day 7.5 (Nasir *et al.* (1995) *Cell* 81, 811-823; Duyao *et al.* (1995) *Science* 269, 407-410; Zeitlin *et al.* (1995) *Nat. Genet.* 11, 155-162). Mice with decreased levels of htt following targeted insertion of a *neo* construct into the *Hdh* gene have aberrant brain development and perinatal lethality (White *et al.* (1997) *Nat. Genet.* 17, 404-410). Mice heterozygous for targeted disruption of the *Hdh* gene (+/-) express half the normal levels of endogenous htt and develop neuronal degeneration in the basal ganglia in adulthood (O'Kusky *et al.* (1999) *Brain. Res.* 818, 468-479). Together, these results suggest that htt may normally be involved in both embryogenesis and in neuronal survival in the adult.

Recently, it was demonstrated that wild-type huntingtin has anti-apoptotic properties *in vitro* (Rigamonti *et al.* (2000) *J. Neurosci.* 20, 3705-3713). To assess the ability of wild-type huntingtin to modulate the toxicity of mutant huntingtin *in vivo*, a transgenic mouse model was developed in which the phenotypic effects of varying endogenous huntingtin levels on mice expressing transgenic huntingtin can be assessed.

Yeast artificial chromosome (YAC) mice were produced that are transgenic for the entire genomic region of the human *HD* gene, including all its regulatory sequences (Hodgson *et al.* (1999) *Neuron* 23, 181-192). Human huntingtin is expressed appropriately during development in YAC transgenic mice as demonstrated by the ability of the human transgene to rescue the embryonic lethality of *Hdh* nullizygous mice (-/-) (Hodgson *et al.* (1996) *Hum. Mol. Genet.* 5, 1875-1885). Appropriate control of tissue specificity was confirmed by the identical expression patterns of endogenous and human transgenic huntingtin by Western blot analysis and subcellular localization studies (Hodgson *et al.* (1996) *Hum. Mol. Genet.* 5, 1875-1885; Hodgson *et al.* (1999) *Neuron* 23, 181-192).

YAC transgenic mice have been generated that express human htt with 18 polyglutamines (YAC18) corresponding to a CAG repeat length observed in unaffected persons, 46 polyglutamines (YAC46) corresponding to a CAG repeat length observed in adult-onset HD patients, and 72 polyglutamines (YAC72) corresponding to a repeat length causing juvenile-onset HD (Hodgson *et al.* (1999) *Neuron* 23, 181-192). These mice express similar levels of transgenic human huntingtin differing only in polyglutamine expansion length. YAC18 mice have no observable phenotype up to 24 months of age, indicating that human huntingtin with a polyglutamine tract of normal length is not pathogenic in mice. However, mice transgenic for mutant huntingtin with an expanded polyglutamine develop a progressive phenotype characterized by behavioral, cellular and neuropathologic abnormalities similar to those observed in HD (Hodgson *et al.* (1999) *Neuron* 23, 181-192).

When crossed to the *Hdh* nullizygous background, YAC transgenic mice survive normally into adulthood (designated YAC -/-). These crosses provide a system in which one can examine the role of wild-type huntingtin in modulating the cellular toxicity of mutant huntingtin *in vivo*. In this study we demonstrate that mutant human huntingtin causes increased apoptotic cell death in the testes of transgenic mice expressing no endogenous htt. This pro-apoptotic effect of mutant huntingtin can be completely inhibited by increased levels of murine wild-type huntingtin, providing the first *in vivo* evidence that wild-type huntingtin can reduce the toxicity of mutant huntingtin.

Generation of experimental mice

YAC transgenic mice (FVB/NJ strain) from lines 29 (YAC18), 668 (YAC46), and 2511 (YAC72) were bred with mice heterozygous for targeted disruption of the endogenous mouse *Hdh* gene (C57BL6 strain) to produce F1 generation hybrid mice. F1 hybrid mice positive for the YAC transgene and heterozygous for the *Hdh* gene were then bred to produce litters of F2 experimental mice as previously described (Hodgson *et al.* (1996) *Hum. Mol. Genet.* 5, 1875-1885). All the F2 offspring of these matings were genotyped and used to generate experimental data. We also bred selected F2 mice to examine mating behavior, breeding success rates and to obtain post-coital sperm counts.

Genotyping was performed by standard PCR based techniques on genomic DNA from tail clippings prepared by phenol-chloroform extraction (Hodgson *et al.* (1996) *Hum. Mol. Genet.* 5, 1875-1885). Protein expression was determined by Western blot in which 200 ug of total protein from homogenized testes was loaded onto a low-bis acrylamide gel, run at 100V for 2 hours, and 200V for 3 hours before being transfereed to PVDF membranes. Blots were probed with anti-huntingtin antibody (HD3 @ 1/1000, Gutekunst *et al.* (1999) *J. Neurosci.* 19, 2522-2534) and detected using ECL (Amersham).

Fertility, mating behavior, and sperm analysis

To assess breeding success, adult male mice of each genotype were placed in breeding cages with single FVB/NJ female mice for at least four months (minimum of three male mice tested per genotype). The total duration of time spent in breeding cages and the total number of live-born litters was recorded for each mouse. Failure to produce any litters after a cumulative duration of breeding of at least 4 months was considered to represent male infertility. New wild-type females with previous successful breeding experience were placed in breeding cages of infertile males to control for any contribution of the female partner. YAC72 -/- (mice expressing human htt with 72 polyglutamines and no endogenous murine htt), YAC72 +/- (mice expressing human htt with 72 polyglutamines on a background of half the normal levels of endogenous murine htt), and YAC72 +/+ (mice expressing human htt with 72 polyglutamines on a background of normal levels of endogenous murine htt) male mice were placed in breeding cages with pseudo-pregnant FVB/NJ females to assess male sexual behavior and post-coital sperm counts. These wild-type female mice were injected with 0.1 ml of pregnant mare serum (Sigma) 48 hours

prior to stimulation with 10 IU of hcg (Sigma) and placement in breeding cages with the transgenic males. Mounting behavior of male mice was scored for 2-3 hours following placement in breeding cage and plug formation was determined by manual inspection of the female mice the following morning.

Post-coital sperm counts were determined from the extracted uterus of all female mice who had evidence of plug formation following breeding with male transgenic mice. Following breeding the plugged female mice were anaesthetized, the uterus and oviducts removed in toto and gently opened in a sterile 12 well tissue culture plate. 0.5 ml of sterile saline was used to flush the uterus, the resultant solution was collected, and examined microscopically for presence of sperm. To obtain quantitative sperm counts, the testes and epidydimi of YAC72 +/+, YAC72+/-, and YAC72-/- mice (n=6 mice per genotype) were removed and immediately weighed. The tissues were then sectioned and placed in tubes containing 0.5 ml of sterile saline. Total numbers of morphologically normal sperm were manually counted using a brightline hemacytometer (Hausser) for three samples from each tissue. The hemacytometer counts for each tissue were averaged, and the total counts per ml were calculated. Results are expressed as average sperm count per ml +/- SEM and significance was determined by the Students t-test.

Histological and Ultrastructural Analysis

Testes were removed from anesthetized animals and immediately placed in fixative (1.5% paraformaldehyde, 1.5% glutaraldehyde, 0.1 M Na cacodylate, pH 7.3). The capsules were nicked with a scalpel and then the organs left to fix for approximately 1 hour. The testes were cut into small pieces and fixed for an additional hour. The pieces were washed with buffer, post-fixed in buffered 1% OsO_4 on ice for 1 hour, washed with dH_20 , and stained en bloc in 1% aqueous uranyl acetate. The samples were washed with dH_20, dehydrated through a graded concentration series of ethyl alcohols, and then embedded in JEMBED™ 812 (J. B. EM Services Inc., Point-Claire, Quebec).

For histological analysis, thick sections (1μm) were stained with toluidine blue and evaluated on a Zeiss Axiophot microscope. For ultrastructural analysis, thin sections were cut on an ultramicrotome, stained with lead citrate and uranyl acetate, and then viewed and photographed on a Philips 300 electron microscope operated at 60 kV.

TUNEL™ labelling (Boehringer Mannheim) was performed using standard techniques on frozen sections of testes that were lightly immersion fixed in 3 % paraformaldehyde. Following removal of the testes, mice were injected with heparin and transcardially perfused with 3% paraformaldehyde and 0.15% glutaraldehyde in phosphate buffer (pH 7.4). Brains were then removed and post-fixed in 3% paraformaldehyde overnight.

Immunocytochemistry

Testes were excised from anesthetized animals, the capsules cut open with a scalpel, and the organs immersion fixed (3% paraformaldehyde, 150mM NaCl, 5 mM KCl, 3.2 mM Na_2HPO_4, 0.8 mM KH_2PO_4, pH 7.3) for one to two hours. Following fixation, the testes were washed three times (10 minutes each wash) with PBS (150mM NaCl, 5 mM KCl, 3.2 mM Na_2HPO_4, 0.8 mM KH_2PO_4, pH 7.3) and then frozen in OCT compound and sectioned on a cryostat. Sections (10 μm thick) were collected on polylysine-coated slides and then the slides were immediately plunged into cold acetone (-20°C) for 5 minutes. Following this, the slides were air-dried.

Sections were re-hydrated for 30 minutes in TPBS (PBS, 0.05% Tween-20, 0.1% BSA) containing 5% normal goat serum (NGS), and then incubated for 1 hour at 37°C with primary antiserum diluted 1:100 (HD3) with TPBS containing 1% NGS. Sections were washed (three times 10 minutes each wash) with TPBS, and then incubated for 1 hour at 37°C with secondary antibody (goat anti-rabbit conjugated to Texas red) diluted 1:100 in TPBS. Sections again were washed with TPBS, mounted in Vetashield™ (Vector Laboratories, Burlingame, CA), and viewed on a Zeiss Axiophot microscope fitted with the appropriate fluorescence filter sets. Controls consisted of replacing the primary antibody with the same concentration of normal rat IgG (control for primary antibody), replacing the primary antibody with buffer alone (control for secondary antibody), and replacing the primary and secondary antibodies with buffer alone (control for autofluorescence).

Rescue of the Hdh -/- lethal phenotype by YAC transgenes expressing mutant human huntingtin

We generated yeast artificial chromosome (YAC) transgenic mice expressing normal (YAC18) or mutant (YAC46 or YAC72) human huntingtin in the absence of endogenous

mouse htt (Hdh -/-). Figure 11a demonstrates the genotype of several offspring from a cross between two mice heterozygous for targeted disruption of the Hdh gene, one of which also carried the YAC72 transgene (YAC72+/-). Mice with targeted disruption of both alleles of the Hdh gene can be rescued from the embryonic lethal phenotype by the YAC transgene expressing mutant huntingtin with 72 CAG repeats (YAC72-/- mice). In this litter, mice were generated with the YAC72 transgene and either 100% of the normal level of endogenous htt (YAC72+/+), 50% of the normal level of endogenous htt (YAC72+/-), or complete absence of endogenous htt (YAC72-/-). A mouse lacking the YAC72 transgene but heterozygous for endogenous huntingtin is also shown (Figure 11A, -,+/-). No Hdh -/- mice were generated in the absence of the YAC transgene, consistent with the previous finding that *Hdh* nullizygous mice are not viable (Nasir *et al.* (1995) *Cell* **81**, 811-823; Duyao *et al.* (1995) *Science* **269**, 407-410; Zeitlin *et al.* (1995) *Nat. Genet.* **11**, 155-162). The F2 offspring of our experimental breedings had the expected 1:2:1 ratio of genotypes for all of the YAC transgenes examined (Figure 11B), demonstrating that both the normal (YAC18) and mutant (YAC46 and YAC72) human HD transgenes compensated for the lack of endogenous murine htt in *Hdh* -/- mice equally.

Huntingtin expression levels in YAC transgene rescued Hdh -/- mice

Levels of the transgenic and wild-type huntingtin protein for YAC18 (line 29), YAC46 (line 668), and YAC72 (line 2511) Hdh -/- mice were measured by Western blot using an antibody recognizing both human and mouse htt (Hodgson *et al.* (1996) *Hum. Mol. Genet.* 5, 1875-1885). The human transgenic protein is expressed at similar levels in the YAC18, YAC46, and the YAC72 mice used in these experiments (Figure 11C). This result was replicated in three different Western blots, and densiometric quantification of the transgenic protein level from these blots revealed a 1:1 ratio of transgenic versus endogenous htt levels in these mice. Transgenic protein levels from YAC18 mice averaged 102% of wild-type htt, YAC46 averaged 99% of wild-type htt, and YAC72 averaged 100% of wild-type htt. These 3 lines of YAC transgenic mice were selected for these experiments because they expressed identical levels of transgenic protein, differing only in the length of the CAG repeat.

Mice expressing mutant huntingtin are infertile

Expression of mutant (YAC46 and YAC72), but not wild-type (YAC18), transgenic huntingtin in the absence of endogenous htt leads to a novel phenotype that was initially identified by the observation that male Hdh -/- mice were unable to breed. We attempted to breed YAC46-/- and YAC72-/- males with wild-type females for extended periods with no success (no offspring were generated). Female littermates with the same genotype (YAC46-/- and YAC72-/-) had normal fertility when bred with wild-type mice. Male YAC72-/- mice had normal secondary sexual characteristics and displayed identical sexual behavior (mounting) and libido as YAC72+/- and YAC72+/+ mice when placed in cages with female wild-type mice. Similar plug formation rates were obtained for males of all three genotypes. Plugs were recovered in 8 of 12 breeding trials for YAC72-/- mice, 7 of 12 breeding trials for YAC72+/- mice and 8 of 16 breeding trials of YAC72+/+ mice. In sharp contrast to both YAC72+/+ and YAC72+/- mice, no post-coital sperm was ever recovered from plugged females following mating with YAC72-/- mice. These results suggested that a defect in spermatogenesis and not breeding behavior was responsible for the observed lack of fertility of male YAC72-/- mice.

Decreased fertility in mice expressing mutant huntingtin is a result of decreased sperm production

To assess spermatogenesis directly in YAC72-/- mice, we performed sperm counts and examined the testes of these mice. YAC72-/- mice had significantly decreased epididymal sperm counts compared to YAC72+/+ (11×10^3/ml ± 2.9 vs. 13×10^5/ml ± 100; $p<0.00001$) or YAC72 +/- (11×10^3/ml ± 2.9 versus 14×10^5/ml ± 65; $p<0.00001$) littermates at 4 months of age (Figure 11D).

Testicular atrophy and spermatid degeneration in mice expressing mutant huntingtin

YAC72-/- mice had significant testicular atrophy compared to YAC72+/+ (average testes weight, 54.0 mg ± 2.9 vs. 84.7 mg ± 3.6.9; $p<0.00001$) and YAC72+/- (average testes weight, 54.0 mg ± 2.9 vs. 90.1 mg ± 3.0; $p<0.00001$) littermates at 4 months of age (Figure 11E).

Histological examination of sections from the testes of adult mice expressing expanded mutant htt (YAC46-/- or YAC72-/-) stained with toluidine blue revealed massive

disruption of spermatogenesis in the seminiferous tubules. The seminiferous tubules from these mice were full of large vacuoles and dying cells (Figure 12 A and D), but the spermatagonial stem cells and Sertoli cells close to the basement lamina appeared relatively normal in appearance and number. Depletion, but not absence, of cells at later stages of spermatogenesis (spermatocytes and spermatids) was evident in all layers of degenerating tubules. Degenerating cells at various stages of development were identified, suggesting that the spermatogenic defect caused by mutant htt is not limited to a single stage of development or due to defective maturation of spermatocytes. The normal stratified organization of cells within these seminiferous tubules was completely disrupted. Rarely, late spermatids were identified in the outer cell layer, but mature spermatazoa were not found in the lumen of these tubules, which were often filled with cellular debris (Figure 12, A and D). Leydig cells appeared to be unaffected in the stromal interstitial tissue between degenerating tubules.

Cellular degeneration in mice expressing mutant huntingtin can be blocked by expression of endogenous htt and is CAG-length dependent

This testicular degeneration was most striking in the testes of YAC72 -/- mice (Figure 12A). Occasionally vacuolisation and cellular degeneration was seen in the testes from YAC72+/- mice expressing 50% of endogenous htt levels (Figure 12B), but these mice were able to produce mature sperm (Figure 11C). Increasing endogenous htt expression to 100% of normal levels in YAC72 mice completely rescued the degenerative testicular phenotype (Figure 12C). Normal stratified organisation was restored to the seminiferous tubules, and no vacuolization or increased numbers of degenerating cells was present. Mature spermatozoa were found in the lumen of seminiferous tubules from YAC72+/+ mice (Figure 12C, arrow) and these mice had normal fertility. These results suggest that the testicular cell death caused by the expression of polyglutamine expanded huntingtin in transgenic mice could be completely blocked by increasing the level of endogenous huntingtin.

The testicular cell death caused by the expression of polyglutamine expanded htt in transgenic mice (YAC46 -/- and YAC72-/-) mice does not occur in mice expressing the same human transgene without the CAG repeat expansion (YAC18 -/- mice, Figure 12G). YAC18 mice lacking endogenous htt had normal morphology of the seminiferous tubules,

no evidence of increased testicular cell death, and normal fertility. No significant effect was seen on YAC18 mice when levels of endogenous htt were increased to 50 or 100% of the normal huntingtin levels (Figure 12 H and I).

Histological examination of toluidine blue stained sections from the testes of adult mice expressing mutant htt revealed massive cellular death in multiple layers of the seminiferous tubules (Figure 13A). TUNEL™ labelling (Figure 13B, arrows) confirmed apoptotic nature of the widespread cell death in the testes of YAC72-/-. Despite the drastically reduced numbers of cells within seminiferous tubules from the YAC72-/- testes, the average number of TUNEL positive cells (3.2 per 20× field) was ~10-fold higher in these sections than in sections from YAC72+/+ testes containing normal numbers of spermatogenic cells (0.3 per 20× field). No increased testicular cell death was observed in YAC72 mice expressing 100% of normal levels of endogenous huntingtin (YAC72 +/+) either by toluidine blue (Figure 13C) or by TUNEL™ labelling (Figure 13D).

Ultrastructural analysis (EM) of the testes in YAC72 mice nullizygous for endogenous huntingtin (-/-) revealed large numbers of degenerating spermatids with diffuse cytoplasmic vacuolisation (Figure 13E). Shrunken degenerating spermatids, with condensed nuclei and electron dense cytoplasm, were phagocytosed and degraded by Sertoli cells (Figure 13F), suggestive of ongoing apoptosis and confirming the TUNEL™ findings. Multinucleated giant cells were found throughout the testes of YAC72-/- mice. These cells result from the opening of intercellular bridges between clones of spermatogenic cells. Importantly, no such degenerative phenotype was found by ultrastructural analysis of the testes of YAC18 mice (data not shown).

Abnormal protein aggregates occur in the testes of mice expressing mutant huntingtin

Ultrastructural analysis of the testes of YAC72 -/- mice revealed the presence of occasional abnormal aggregates of intracellular protein (arrows) within spermatids (Figure 14A), Sertoli cells (Figure 14B), and sperm tails (Figure 14C). These aggregates were rare, and found at an incidence of much less than one per high-powered field. No protein aggregates were identified in in YAC18-/- mice (data not shown). Ectopic microtubule bundles (Figure 14D) and manchettes (Figure14E) were also occasionally identified

(arrows) within spermatogonia and spermatids respectively. Also interesting is the observation that actin-containing adhesion plaques (ectoplasmic specializations) that occur in Sertoli cell cortical cytoplasm in regions of adhesion to spermatids often occurred in ectopic positions. Normally these structures occur in regions of attachment anly to spermatid heads. In YAC72 -/- Sertoli cells, ectoplasmic specializations were observed to completely surround elongate spermatids that had re-acquired a circular form (data not shown).

Immunocytochemical analysis of huntingtin localization in the testes of YAC72-/- mice revealed that protein aggregates within a small numbers of degenerating spermatids (Figure 15, A and B) contain huntingtin. Similar huntingtin immunoreactivity was identified in aggregates within Sertoli cells adjacent to the basal lamina of degenerating seminiferous tubules (Figure 15, C and D). Labelling of filamentous actin with fluorescent phallotoxin revealed altered localization of filaments within the testes of YAC72-/- mice (Figure 15, I and J). In YAC72 +/+ (Figure E and F), actin filaments were concentrated in Sertoli cell adhesion plaques (ectoplasmic specializations) found apically in association with spermatid heads and basally in association with junction complexes between neighbouring Sertoli cells. In YAC72-/- tissue (Figure 15, I and J) actin filaments occur in linear arrays perpendicular to the tubule wall and in areas not directly related to spermatid heads (arrows with asterisks in Figure 15, J).

The data herein provides evidence that wild-type huntingtin can significantly reduce the cellular toxicity of mutant huntingtin *in vivo*. Expression of human huntingtin with an expanded polyglutamine tract (46 and 72 polyglutamines) in the absence of wild-type htt results in male infertility, and massive apoptotic cell death in the testes in all phases of spermatogenesis. The cell death can be modulated by the expression of normal huntingtin. For example, mice expressing human huntingtin with 46 or 72 polyglutamines have no evidence for testicular atrophy or apoptosis in the testes when wild-type huntingtin is expressed from both *Hdh* alleles in the mouse. An intermediate phenotype is seen in these mice (YAC46 and 72) on the background of heterozygosity for targeted disruption in the mouse *Hdh* gene (YAC46 +/- or YAC72 +/-). The severity of the testicular atrophy and apoptotic cell death is also modulated by the length of the polyglutamine repeat. YAC72 transgenic mice require higher levels of wild-type htt (+/+) than YAC46 transgenic mice

(+/-) to prevent testicular degeneration. Abnormal protein aggregates, that contain htt, are occasionally found both in Sertoli cells and in spermatogenic cells in the testis of YAC72-/- mice. Also, structures containing cytoskeletal elements form ectopically in these mice suggesting that alterations in either the targeting of cytoskeletal elements to specific positions in the cells or of cytoskeletal function may be a mechanism promoting the massive apoptosis observed. These findings suggest that disruption of normal cytoskeletal organization may play a role in mediating the toxic effect of mutant huntingtin.

Both mutant and wild-type huntingtin proteins undergo cleavage (Wellington *et al.* (1999) *J. Biol. Chem.* 273, 9158-9167), and are recruited and sequestered into htt aggregates leaving less full-length wild-type huntingtin available to counteract pro-apoptotic stimuli (Martindale *et al.* (1998) *Nat. Genet.* 18,150-154). Polyglutamine expansion in one allele of the *HD* gene is associated with expression of half the cellular levels of wild-type huntingtin compared to normal neurons, which, together with huntingtin cleavage and sequestration of wild-type huntingtin in aggregates, may further decrease functional huntingtin levels. Mice heterozygous for targeted disruption of the *Hdh* gene express half the normal levels of wild-type huntingtin, and have previously been shown to develop neuronal degeneration in the basal ganglia (Nasir *et al.* (1995) *Cell* 81, 811-823; O'Kusky *et al.* (1999) *Brain Res.* 818, 468-479). One of the normal functions of huntingtin in the brain may be to protect cells against pro-apoptotic stimuli, and partial loss of this function may underlie some of the selective vulnerability of striatal neurons to cell death in HD. These data are in support of our previously stated hypothesis that loss of function of wild-type htt may also contribute to the pathogenesis of HD ((Nasir *et al.* (1995) *Cell* 81, 811-823).

Wild-type human huntingtin can protect hippocampal neurons from kainic acid-induced excitotoxicity . Furthermore, the very recent report that inactivation of *Hdh* expression in adult mice is associated with progressive apoptotic neurodegeneration provides further support for the anti-apoptotic role of wild-type huntingtin in the adult brain (Dragatsis *et al.* (2000) *Nat. Genet.* 26, 300-306).

A number of observations indicate that the ultimate outcome of huntingtin toxicity in the testis is the apoptotic loss of spermatogenic cells. The dramatic vacuolisation within the

seminiferous tubules, TUNEL™ staining of spermatogenic cells, and the ectopic positioning of manchettes (microtubule structures associated with spermatid nucleii) in these cells are consistent with this conclusion. The presence of giant cells in the epithelium, the occurrence of large phagosomes containing spermatids in Sertoli cells and the obviously reduced numbers of spermatogenic cells in the epithelium demonstrated by our EM analysis all point to the conclusion that the primary testicular phenotype in YAC72 -/- mice is apoptotic death of spermatogenic cells, particularly of spermatids.

Although morphological changes and cell loss are most dramatic in the spermatogenic cell population in YAC72-/- testes, Sertoli cells also express abnormal features. Two of these features are the presence of protein aggregates in the cytoplasm and the ectopic positioning of actin filament containing junction plaques normally found in regions adjacent to spermatid heads. Abnormal positioning of ectoplasmic specializations adjacent to spermatids cells may be a response to a primary defect in the associated spermatogenic cells. The presence of normally positioned junction plaques in basal regions of attachment to neighbouring Sertoli cells is consistent with this conclusion. These findings suggest that mutant huntingtin causes an intrinsic polyglutamine-mediated apoptotic cell death within spermatogenic cells, which can be blocked by wild-type huntingtin.

It has previously been shown that human huntingtin can compensate for the critical function of murine huntingtin during gastrulation by rescuing mice with targeted disruption on both *Hdh* alleles studies (Hodgson *et al.* (1996) *Hum. Mol. Genet.* 5, 1875-1885). These mice are rescued by both normal (YAC18) and mutant huntingtin (YAC46 and YAC72), clearly indicating that expansion of the polyglutamine does not disturb this important role of huntingtin in development. Here we have provided further evidence for cross-species functional complementarity of huntingtin. The presence of murine huntingtin can completely protect cells against the pro-apoptotic effects of mutant human huntingtin. Cross-species functional complementation between mouse and human huntingtin in development and in protection against apoptosis must reflect their high degree of sequence conservation with mouse and human huntingtin sharing complete identity of nucleotides at a level of 90% similarity in amino acid structure (Lin *et al.* (1994) *Genomics* 25, 707-715.

The data provided herein demonstrates strong *in vivo* evidence that wild-type huntingtin can significantly modulate the apoptotic toxicity of mutant huntingtin, and we suggest that wild-type huntingtin may normally have an anti-apoptotic function. Mapping the critical region of huntingtin responsible for this function and investigation of the mechanism by which this critical region influences cell death pathways may identify novel therapeutic targets for HD and advance our understanding of huntingtin's normal role in the delicate balance between life and death in cells.

Role of wild-type huntingtin on cell proliferation and tumor formation in vivo.

One of the roles of the pro-survival (e.g. anti-apoptotic) function of wild-type huntingtin may be in control of tissue mass and cellular proliferation. Increased levels of wild-type htt expression can lead to increased body tissue mass and may predispose mice to the development of tumors. The effect of over-expression of human wild-type huntingtin on the body weight of transgenic mice was compared to wild-type mice (Figure 16). A significant increase in body weight was observed in transgenic mice compared to control mice, suggesting that increasing wild-type huntingtin function increases the number of cells in the body. Over production of cells in the body can have a variety of unwanted outcomes including but not limited to obesity or cancer. This effect could be a result of a decreased amount of naturally occurring apoptosis or increased cellular proliferation.

A retrospective analysis was performed to examine the total numbers of mice (identified by the animal colony health staff) that developed tumours in the colony of transgenic mice. The records of all mice diagnosed with tumours over a 6 month period were examined, in a group of mice over-expressing wild-type human huntingtin and their wild-type littermates. The vast majority of mice that developed tumours were found to be transgenic mice that over-expressed wild-type huntingtin (38/45 or 84.5 % of mice identified with tumours were transgenic). The average age of tumour development was younger in the transgenic group 13.8 months compared to 17.1 months for wild-type littermates. The majority of identified tumours in male mice were testicular tumours. Huntingtin is expressed at very high levels in the testes suggesting that this tissue may be at increased risk of tumour development when huntingtin levels are increased.

Role of huntingtin on cell proliferation in vitro.

Huntingtin is required for the normal cellular proliferation of hematopoetic cells (Metzler *et al.* (2000) *Hum. Mol. Genet.* 9, 387-394). In these experiments embryonic stem cells that had decreased levels or complete absence of huntingtin were exposed to hematopoetic cytokines that stimulate cellular proliferation and found to have less response (less proliferation) than stem cells that had the normal levels of huntingtin. This early data suggests that huntingtin can have both anti-apoptotic and proliferative functions.

The ability of NIH3T3 cells to overcome contact inhibition when transfected with different gene constructs has been used for many years to assess oncogenic potential of certain genes and to identify potential oncogenes (genes that cause cancer). Using a standard screen of cellular proliferation in NIH3T3 cells, the effect that huntingtin has on cellular growth was examined and compared to the effect of known oncogenes (Figure 17). NIH3T3 cells transfected with huntingtin constructs show a significant increase in cellular proliferation (5-6 fold increase in proliferation compared to control vector) that is similar to the effect of a well-described oncogene, *ras*.

The *in vitro* and *in vivo* data demonstrates that the anti-apoptotic function of huntingtin plays a role in controlling cellular proliferation and that alterations in this function can cause cancer or neurodegeneration depending on whether huntingtin function is increased or decreased respectively.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing form the true scope of the invention and appended claims.

Table 1: Offspring of HD Knock-out Rescue Breedings

	+/+	+/-	-/-
YAC18	19	33	15
YAC46	17	60	31
YAC72	9	8	4
Total:	45	101	49

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of regulating cell survival comprising the step of altering levels of huntingtin function.

2. Use of a protein according to the method of claim 1, to modulate cell death or cell proliferation in a mammal having a condition characterised by a dysregulation of cell death or cell proliferation, wherein said protein is selected from the group consisting of huntingtin and a biologically active fragment of huntingtin protein.

3. Use of a nucleic acid encoding huntingtin, or a biologically active fragment thereof, according to the method of claim 1, to modulate cell death or cell proliferation in a mammal having a condition characterised by a dysregulation of cell death or cell proliferation.

4. Use of an antagonist of huntingtin according to the method of claim 1, to modulate cell proliferation in a mammal having a condition characterised by a dysregulation of cell proliferation.

5. The use according to claim 2 or 3, wherein said protein inhibits cell death.

6. The use according to any one of claims 2, 3 or 4, wherein said protein inhibits cell proliferation.

7. The use according to any one of claims 2, 3 or 5, wherein said condition is a neurodegenerative disease.

8. The use according to claim 7, wherein said neurodegenerative disease is Huntingtin's disease, any other polyglutamine disorder, Alzheimer's disease, amyotrophic lateral sclerosis or Parkinson's disease.

9. The use according to any one of claims 2, 3, 4 or 6, wherein said condition is cancer.

10. The use according to claim 9, wherein said cancer is a germ cell cancer.

11. The use according to claim 10, wherein said germ cell cancer is testicular cancer.

12. The use according to claim 4, wherein said antagonist is an antisense oligonucleotide.

13. The use according to claim 4, wherein said antagonist is an anti-huntingtin antibody.

14. The use according to claim 4, wherein said antagonist is a small molecule that binds to huntingtin or to a nucleic acid encoding huntingtin.

15. A nucleic acid encoding a biologically active fragment of huntingtin protein.

16. An antagonist of huntingtin.

17. The antagonist according to claim 16 which is an antisense oligonucleotide.

18. The antagonist according to claim 16 which is an anti-huntingtin antibody.

19. The antagonist according to claim 16 which is a small molecule that binds to huntingtin or to a nucleic acid encoding huntingtin.

20. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and huntingtin, a biologically active fragment of huntingtin, or a combination thereof.

21. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a nucleic acid encoding huntingtin, a nucleic acid encoding a biologically

active fragment of huntingtin, or a combination thereof.

22. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and an antagonist of huntingtin.

23. The pharmaceutical composition according to claim 22 which is an antisense oligonucleotide.

24. The pharmaceutical composition according to claim 22 which is an anti-huntingtin antibody.

25. The pharmaceutical composition according to claim 22 which is a small molecule that binds to huntingtin or to a nucleic acid encoding huntingtin.

26. Use of huntingtin, a biologically active fragment of huntingtin, or a combination thereof for the preparation of a medicament for the treatment of conditions characterised by dysregulation of cell death or cell proliferation.

27. Use of a nucleic acid encoding huntingtin or a biologically active fragment thereof, for the preparation of a medicament for the treatment of conditions characterised by a dysregulation of cell death or cell proliferation.

28. Use of an antagonist of huntingtin for the preparation of a medicament for the treatment of conditions characterised by a dysregulation of cell proliferation.

29. The use according to claim 28, wherein said antagonist is an antisense oligonucleotide.

30. The use according to claim 28, wherein said antagonist is an anti-huntingtin antibody.

31. The use according to claim 28, wherein said antagonist is a small molecule that binds to huntingtin or to a nucleic acid encoding huntingtin.

32. An assay for screening for molecules having an anti-proliferative activity comprising the steps of:

(a) transfecting NIH3T3 cells with huntingin;

(b) culturing the transfected cells in the presence and absence of a candidate molecule;

(c) comparing proliferation of the transfected cells in the presence of the candidate molecule with proliferation of the transfected cells in the absence of the candidate molecule,

wherein an anti-proliferative activity is identified a decrease in the proliferation of the transfected cells in the presence of the candidate molecule in comparison to the proliferation of the transfected cells in the absence of the candidate molecule.

33. The assay according to claim 32, wherein in step (b) the transfected cells are cultured in a state of contact inhibition.

34. The assay according to claim 32, wherein in step (b) the transfected cells are cultured in soft agar.

35. The assay according to claim 32, wherein in step (b) the transfected cells are cultured in an animal.

36. The assay according to claim 35, wherein in said animal is a mouse.

RAM/PCT

JONHSON CONTROLS

DATE 12/20/01

SERIAL NUMBER 10/009498

☐ DIFFERENT SERIAL NUMBER

☐ (----------) HAD INSUFFICIENT FUNDS

☐ DEPOSIT ACCOUNT IS NOT FOUND

☐ NO SIGNATURE

☒ NO AUTHORIZATION

☐ USER IS NOT LISTED

☐ CREDIT CARD DECLINE

☐ NO CALCULATION SHEET

OPERATED BY: pmw

Copy for the designated Office (DO/US)

PCT/CA01/00495

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MBM & CO.
P.O. Box 809
Station B
Ottawa, Ontario K1P 5P9
CANADA

Date of mailing (day/month/year)
18 February 2002 (18.02.02)

Applicant's or agent's file reference
338-110PCT

IMPORTANT NOTIFICATION

International application No.
PCT/CA01/00495

International filing date (day/month/year)
12 April 2001 (12.04.01)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
UNIVERSITY OF BRITISH COLUMBIA
Industry Liaison Office
111-2386 East Mall
Vancouver, British Columbia V6T 1Z3
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State of Nationality: CA
State of Residence: CA
Telephone No.: 1-604-822-8580
Facsimile No.: 1-604-822-8589
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
UNIVERSITY OF BRITISH COLUMBIA
2194 Health Sciences Mall
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State of Residence: CA
Telephone No.: 1-604-822-8580
Facsimile No.: 1-604-822-8589
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[X] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
P. Blanchet (Fax 338.87.40)

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

004668973

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PCT/CA01/00495

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION RELATING TO PRIORITY CLAIM

(PCT Rules 26bis.1 and 26bis.2 and Administrative Instructions, Sections 402 and 409)

To:

MBM & CO.
P.O. Box 809
Station B
Ottawa, Ontario K1P 5P9
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 19 September 2001 (19.09.01)	
Applicant's or agent's file reference 338-110PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/CA01/00495	International filing date (day/month/year) 12 April 2001 (12.04.01)
Applicant UNIVERSITY OF BRITISH COLUMBIA et al	

The applicant is hereby **notified** of the following in respect of the priority claim(s) made in the international application.

1. [X] **Correction of priority claim.** In accordance with the applicant's notice received on: 29 August 2001 (29.08.01), the following priority claim has been corrected to read as follows:

CA 12 December 2000 (12.12.00) 2,326,543

- [ ] even though the indication of the number of the earlier application is missing.
- [ ] even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

2. [ ] **Addition of priority claim.** In accordance with the applicant's notice received on: , the following priority claim has been added:

- [ ] even though the indication of the number of the earlier application is missing.
- [ ] even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

3. [ ] As a **result of the correction and/or addition** of (a) priority claim(s) under items 1 and/or 2, the (earliest) priority date is:

4. [ ] **Priority claim considered not to have been made.**
   - [ ] The applicant failed to respond to the Invitation under Rule 26bis.2(a) (Form PCT/IB/316) within the prescribed time limit.
   - [ ] The applicant's notice was received after the expiration of the prescribed time limit under Rule 26bis.1(a).
   - [ ] The applicant's notice failed to correct the priority claim so as to comply with the requirements of Rule 4.10.

   The applicant may, before the technical preparations for international publication have been completed and subject to the payment of a fee, request the International Bureau to publish, together with the international application, information concerning the priority claim. See Rule 26bis.2(c) and the PCT Applicant's Guide, Volume I, Annex B2(IB).

5. [ ] In case where **multiple priorities** have been claimed, the above item(s) relate to the following priority claim(s):

6. A copy of this notification has been sent to the receiving Office and
   - [ ] to the International Searching Authority (where the international search report has not yet been issued).
   - [X] the designated Offices (which have already been notified of the receipt of the record copy).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Gabriele BAEHR

Telephone No. (41-22) 338.83.38

004296896

Copy for the designated Office (DO/US)

PCT/CA01/00495

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION RELATING TO PRIORITY CLAIM

(PCT Rules 26bis.1 and 26bis.2 and Administrative Instructions, Sections 402 and 409)

To:

MBM & CO.
P.O. Box 809
Station B
Ottawa, Ontario K1P 5P9
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 25 June 2001 (25.06.01)	
Applicant's or agent's file reference 338-110PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/CA01/00495	International filing date (day/month/year) 12 April 2001 (12.04.01)
Applicant UNIVERSITY OF BRITISH COLUMBIA et al	

The applicant is hereby **notified** of the following in respect of the priority claim(s) made in the international application.

1. [X] **Correction of priority claim.** In accordance with the applicant's notice received on: 11 May 2001 (11.05.01), the following priority claim has been corrected to read as follows:

CA 11 December 2000 (11.12.00) 2,326,543

[ ] even though the indication of the number of the earlier application is missing.
[ ] even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

2. [ ] **Addition of priority claim.** In accordance with the applicant's notice received on: , the following priority claim has been added:

[ ] even though the indication of the number of the earlier application is missing.
[ ] even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

3. [ ] As a **result of the correction and/or addition** of (a) priority claim(s) under items 1 and/or 2, the (earliest) priority date is:

4. [ ] **Priority claim considered not to have been made.**
[ ] The applicant failed to respond to the Invitation under Rule 26bis.2(a) (Form PCT/IB/316) within the prescribed time limit.
[ ] The applicant's notice was received after the expiration of the prescribed time limit under Rule 26bis.1(a).
[ ] The applicant's notice failed to correct the priority claim so as to comply with the requirements of Rule 4.10.
The applicant may, before the technical preparations for international publication have been completed and subject to the payment of a fee, request the International Bureau to publish, together with the international application, information concerning the priority claim. See Rule 26bis.2(c) and the PCT Applicant's Guide, Volume I, Annex B2(IB).

5. [ ] In case where **multiple priorities** have been claimed, the above item(s) relate to the following priority claim(s):

6. A copy of this notification has been sent to the receiving Office and
[X] to the International Searching Authority (where the international search report has not yet been issued).
[X] the designated Offices (which have already been notified of the receipt of the record copy).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer F. Baechler
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Form PCT/IB/318 (July 1998)

004108469